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LI. THE MECHANISM OF THE REVERSAL IN REACTION OF A MEDIUM WHICH TAKES PLACE DURING GROWTH OF *B. DIPHTHERIAE*.

By CHARLES GEORGE LEWIS WOLF.

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(*Received June 1st, 1922.*)

A Report to the Medical Research Council.

In the preparation of diphtheria toxin for immunising purposes it is known that the initial and final reactions of the medium are factors of prime importance in harvesting a toxin of high potency. The more recent investigations of L. Davis [1918], Bunker [1918, 1919] and Hartley [1922] have placed this beyond a doubt. Bunker, indeed, goes so far as to say that, outside certain well-defined final hydrogen ion concentrations, potent toxins are never harvested, and for this reason has suggested that a determination of the hydrogen ion concentration by modern methods is a criterion for ascertaining when the organism has elaborated the greatest amount of toxin. Hartley questions this and has been able to show that while Bunker's statement is approximately correct, there are cases in which a very high grade antigen is produced outside the limits set by Bunker.

For the purposes of the present paper it is only necessary to refer to the course which the medium takes when reaching this final reaction.

If one starts with a bouillon peptone medium of $p_H = 7{\cdot}8$, during the earlier period of growth the reaction becomes more acid, often exceeding the neutral point of $p_H = 7{\cdot}0$. A sudden change in reaction occurs when this acid point is reached and the medium becomes alkaline. The alkalinity may reach the high value of $p_H = 8{\cdot}6$ or even $8{\cdot}8$. It is during the period of alkalinity that the toxin is formed.

The chemical reactions which occasion the change to the acid side of neutrality and then to the alkaline side with this bacillus have been little studied and not much is known about them. The acid production early received the attention of Theobald Smith [1895, 1899] who believed that it was closely connected with the presence of glucose in the bouillon. Later on he seems to have modified his view and to have attached more importance to the presence of inositol, the so-called muscle sugar, as a producer of acid, and in certain experiments on toxin production he sought to remove all fermentable carbohydrates by the preliminary treatment of the medium with *B. coli* or

yeast. It is noteworthy that he suggests that after this treatment a small amount of glucose should be added to the medium for the more efficient production of toxin. Smith's experiments have had a very considerable influence on the technique of toxin production. Even as late as 1921, Dernby and David [1921] have recommended the preliminary fermentation of media by yeast before proceeding to the inoculation with the diphtheria bacillus.

Smith's statements regarding glucose in the bouillon were based on amounts of gas obtained in a fermentation tube after sowing the bouillon with *B. coli*, and there does not seem to be any real information available as to the amount of glucose in meats as ordinarily purchased. In a subsequent paper I hope to be able to give some data on this point. For the present one may say that, assuming the amount of glucose to be 0·06 %, this would yield, if completely converted into carbon dioxide by fermentation, 0·088 % by weight of this gas. This expressed as gas per 100 g. of meat at room temperature and normal pressure would be about 47 cc. It is improbable that such a reaction occurs. One might also assume that a complete transformation of glucose to lactic acid took place. In this event, the amount of lactic acid produced would be equivalent to 0·66 cc. of normal lactic acid per 100 cc. of nutrient broth. Wolf and Harris (1917, 2) have shown that by adding this amount of lactic acid to broth the reaction shifted to the acid side to about the same extent as is seen when *B. diphtheriae* acts upon peptone bouillon. As the amount of meat corresponding to the bouillon is at least half the quantity reckoned as above, it seems inevitable to conclude that the acids produced are not derived solely from the fermentation of the sugar contained in the medium.

The only statement regarding the amount of inositol to be found in meat is an early one of O. Jacobsen [1871] who estimated that fresh muscle contained 0·035 %. In any case, so far as I am aware, no controls have ever been made to ascertain the effect of inositol added to a medium which has been fermented with yeast or with *B. coli*.

That there are other views on the origin of the acid appears from the discussion which took place between Madsen [1897] and Lubenau [1908]. The former author contended that the reversal of the medium was determined by the initial reaction. Lubenau asserted that with the absence of fermentable carbohydrates there was never any acid reaction, provided that the growth took place in the presence of air. Jacobsen [1911] attempted to reconcile these two points of view and tried to show that peptone also has a capacity for forming acids. Petruschky [1891] also believed that peptone furnishes material for the production of acid substances.

The probability is that the truth lay rather with Madsen. With a high initial alkalinity and enough efficient buffer substances in the medium, despite the formation of acids, the reaction of the medium would never reach an acid point as determined with litmus.

When it came to the question of reversal, the matter was on less certain

ground. The obvious product in fermentation which can lead to an alkaline reaction is ammonia, and to this was attributed the change to an alkaline reaction.

There were certain indications that this could not be the case. For example, Teruuchi and Hida [1912] examined the daily production of ammonia in 2 % bouillon peptone, with and without the addition of small quantities of glucose. While they obtained a small increase of ammonia, commencing on the fourth day, they did not find that the alkalinity bore any relation to the amount of ammonia produced.

The more recent work of Ayers and Rupp [1918] gives a strong clue to the mechanism whereby the medium becomes alkaline. In their paper on the well-known reversion produced by organisms of the colon-aerogenes group it is shown that, with *B. coli*, acid and alkaline fermentations proceed simultaneously. Glucose is converted to organic acids and these, in turn, are transformed to carbonates. They suggest that the reversion of reaction is probably due to bicarbonates. A similar simultaneous acid and alkaline fermentation has been analysed by Harris and the writer [1917, 1] in the case of strongly proteolytic anaerobic organisms. Here the stabilisation of the reaction, in spite of the production of large amounts of organic acids, is due in great part to the production of ammonia. It seemed, therefore, of some importance to ascertain to what the reversal in reaction with *B. diphtheriae* was due.

The first experiment was designed to follow out the changes in hydrogen ion concentration, and volatile acid and ammonia production. For this purpose portions of 100 cc. of a standard bouillon-peptone medium were sterilised in 450 cc. rectangular medicine bottles. These were incubated lying on their sides, so that the surface of medium exposed to the air was about 115 sq. cm. At stated times a bottle was taken from the incubator and the hydrogen ion concentration, volatile acids and ammonia determined. Table I gives the results of the experiment.

Table I.

Hours	p_H	Mgm. ammonia nitrogen per 100 cc.	Volatile acids, cc. $N/10$ alkali per 100 cc.
Control	7·5	6·13	4·4
24	7·0	7·85	21·2
48	7·1	8·40	24·8
72	7·9	8·98	8·8
96	8·4	8·52	7·2
120	8·5	8·52	6·8
144	8·5	8·42	7·2
168	8·5	8·98	7·8
192	8·4	9·25	8·0
216	8·5	11·20	8·0
240	8·5	10·07	8·0
264	8·5	8·12	6·0

Certain quite clear results emerge from the analyses. Starting with an initial hydrogen ion concentration of $p_H = 7·5$ the reaction follows the usual course, becoming as acid as $p_H = 6·9$. When reversion took place, a final

value of $p_H = 8{\cdot}5$ was reached which persisted as long as the experiment was continued. The amount of ammonia produced is definite but very small. Starting from an initial value of 6·13 mgm. ammonia N per 100 cc. of medium it does, on one occasion, reach a concentration of 11·2 mgm., but the average is somewhat less than this. This experiment confirms what Teruuchi and Hida found, viz. that the amount of ammonia formed during the metabolism of the bacillus is very small indeed. At the same time a very remarkable change takes place in the volatile acid production. Starting with an amount of acid equivalent to 4·4 cc. of $N/10$ NaOH per 100 cc. of medium, in the first 24 hours the acid had risen to 21·2 cc. with a further rise to 24·8 cc. at the end of 48 hours. With the sudden reversal of reaction from $p_H = 7{\cdot}1$ to 7·9 a large proportion of the acid disappears and in the fourth sample only 8·8 cc. are found. From this time on the amount of acid rises only slightly with a final alkalinity of the medium of $p_H = 8{\cdot}5$.

It is thus obvious that a large part of the volatile organic acids formed in the first stages of fermentation are utilised in the sense of Ayers and Rupp to form basic substances.

A second experiment was undertaken to ascertain to what extent the fixed acids took part in the changes above referred to. The results are embodied in Table II.

Table II.

Hours	p_H	CO_2, cc. per 100 cc.	Volatile acids $N/10$ alkali per 100 cc.	Fixed acids $N/10$ alkali per 100 cc.
Control	7·8	3·0	3·6	27·2
24	7·5	—	4·4	20·5
48	7·3	—	14·8	6·6
72	7·4	—	15·4	11·1
96	7·5	—	15·8	7·4
120	8·1	18·0	7·2	4·5
144	8·2	—	6·8	4·1
192	8·5	27·0	7·2	2·9
240	8·5	27·0	7·4	3·5

The initial alkalinity of the medium was greater than in Exp. 1 and possibly for this reason the medium did not become so acid. The final reaction was, however, the same as in the previous test.

As the amount of medium was not sufficient, no analyses for ammonia were performed. The general trend of the volatile acid curve is the same as in the preceding experiment; one finds a sudden increase in the concentration of these compounds corresponding with a decrease in p_H. The increase in alkalinity is coincident with the sudden lowering of the volatile acids. The course of the fixed acid curve is less easy to explain. From the time of inoculation there is a steady fall, only one value—that obtained at 72 hours—being outside the curve.

The main non-volatile organic acid in bouillon is undoubtedly lactic acid, and this appears to have been metabolised, apparently during the earlier hours of the fermentation, to substances still having an acid reaction and it may well

be that some of these are represented in the increase to be found in the volatile acids. Finally, however, the fixed acids reach a level represented by about 4·0 mgm. of $N/10$ alkali per 100 cc. of medium.

During the course of this experiment I was led to ascertain whether the available carbon dioxide in an old culture was different from that in a medium which had not been inoculated. For this work I employed the van Slyke apparatus, using 1·0 cc. of medium for a determination. It appeared at once that the amount in the former was over five times that of the latter.

Using the present series of cultures it was found that at the 120th hour an amount of carbon dioxide was disengaged by dilute sulphuric acid equivalent to 20·0 cc. of gas per 100 cc. of medium. This was at a time when the reaction of the medium was $p_H = 8·1$. When the fluid reached an alkalinity of $p_H = 8·5$ the amount of carbon dioxide disengaged was 29·0 cc. per 100 cc. of medium. The amount of carbon dioxide disengaged from the control medium was only 3·0 cc., and of this 2·0 cc. were due to gases dissolved in the reagents used for the determination.

It would thus appear that the reversal of reaction was closely connected with the formation of carbonates, confirming what Ayers and Rupp had found with *B. aerogenes*. The matter was therefore studied from the commencement of fermentation.

At the same time it was decided to obtain some information regarding the action of *B. diphtheriae* on certain well-known organic salts. Preliminary experiments performed some time previously made it appear that certain organic acids added to the medium would be available as a source of energy, and a detailed examination of this point was made. Not a great deal of information is available on the latter point.

Table III.

	Control			Sodium succinate			Sodium tartrate		
Hours	Pellicle	p_H	CO_2	Pellicle	p_H	CO_2	Pellicle	p_H	CO_2
0		7·8	1		7·8	1		7·8	1
48	+ + +	7·6	10	+ + +	8·0	23	+ + +	7·3	9
72	+ + +	8·1	9	+ + +	8·1	21	+ + +	8·0	17
120	+ + +	8·3	23	+ + +	8·6	57	+ + +	8·2	19
168		8·3	34		8·6	65		7·9	12
240		7·3?	5?	+ + +	8·5	71	+ + +	8·0	11
336		8·3	27		8·6	75		8·2	22
456		8·5	27		8·8	90		8·5	28
624		8·4	21		8·6	56		8·3	13

	Sodium malate			Sodium citrate			Sodium formate		
Hours	Pellicle	p_H	CO_2	Pellicle	p_H	CO_2	Pellicle	p_H	CO_2
0		7·8	1		7·8	1		7·8	1
48	+ + +	7·7	29	0	7·3	4	+ + +	7·5	10
72	+ + +	8·3	50	0	7·2	5	+ + +	7·5	16
120	+ + +	8·6	75	0	7·3	7	+ + +	7·9	20
168		8·6	66		7·5	7		8·3	17
240	+ + +	8·1?	20?	0	7·5	6	+ + +	8·3	20
336		8·8	90		8·3	21		8·4	26
456		8·8	92		8·3	23		8·4	26
624		8·6	71		7·4	3		8·3	19

Maassen [1896] many years ago in an elaborate investigation of the action of bacteria on organic salts found in a series of 21 organic acids that malic acid was the only one attacked by *B. diphtheriae* and that very feebly.

Altobelli [1914] in a somewhat recent paper also states that this acid is the only one attacked by *B. diphtheriae*.

In the present experiments the fermentations were carried out in test-tubes, the quantity of medium used being about 10 cc.

The salts were added in a concentration of about 1·0 %. After the addition of the salts the medium was adjusted to $p_H = 7{\cdot}8$ and sterilised at 120° for 30 minutes. The tubes were all inoculated from the same culture and kept at 37°. The culture used (Park and Williams, No. 8) had been transferred at 24-hour intervals in bouillon peptone and was growing very actively (Table III).

It is clear that various organic acids are utilised in quite a special way by *B. diphtheriae*. The two which are most strongly attacked are malic and succinic acids. Judged by the amount of carbonate formed, succinic acid is nearly as completely used as malic acid and it is difficult to understand how Maassen overlooked its effect on metabolism. Tartaric acid and formic acid do not seem to have been utilised at all, for the amounts of carbonates formed are very close to those given by the control medium. Citric acid appears to have had an inhibiting effect on the growth of the bacillus. Reversal was very much delayed and the alkalinity did not reach the height obtained with other cultures. The carbonate fermentation was below that of the control medium.

A special interest attaches to formic acid, as this compound has been shown to be one of the steps through which the higher acids are finally oxidised to carbon dioxide. In the katabolism of glucose by *B. coli* the intermediary between this compound and carbon dioxide is said to be formic acid [Harden and Penfold, 1912]. This does not appear to be the case with the diphtheria bacillus, for the addition of formic acid to the medium is not associated with an increase in carbon dioxide above that found in the control medium.

Summary.

The reversal of reaction in carbohydrate-free media caused by the growth of *B. diphtheriae* is due, in its acid phase, in part to the production of volatile acids. When the medium reverses to a more alkaline reaction, these acids are converted into carbonates.

Certain organic acids, such as malic and succinic acids, are also utilised to produce carbonates.

The formation of carbonates from the acids examined does not appear to take place with formic acid as an intermediary product.

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LII. THE PROPERTIES OF DIBENZOYLCYSTINE.

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Nearly all the organic substances exhibiting gel formation in water are compounds of complex structure and high molecular weight.

In order to elucidate the mechanism of gel formation, in recent years search has been made for substances of relatively low molecular weight and simple constitution, and the dye-stuffs, benzo-purpurin, chrysophenone, thionin blue, the soaps, the organic sulphonic acids and camphorylphenylthiosemicarbazide have been used. As Gortner and Hoffman have recently pointed out [1921], benzoylcystine is the simplest known type of organic substance which exhibits gelation with water and it therefore appeared to present a suitable material with which to study some of the elementary properties of gels in greater detail.

The preparation of dibenzoylcystine used in this work was essentially that of Brenzinger [1892] and Gortner and Hoffman [1921], except that the substance, after recrystallisation from dilute alcohol, was extracted for several hours with benzene in a Soxhlet apparatus to remove completely all traces of benzoic acid and benzoyl chloride. The melting-point of our product was 189° (uncorrected) which is some eight degrees higher than that given by the older authors.

While the method of dispersion in water from an alcoholic solution is well known, yet for many purposes the presence of a second component is undesirable, and accordingly attempts were made to obtain direct dispersion of the material in water. It was found that aquagels may be prepared by triturating the solid in small quantities at a time with warm water and completing peptonisation by stirring the solution for long periods at a temperature of 80°–90°. The rigidity of the gels obtained by this method was essentially of the same order as that of those described by Gortner and Hoffman using alcohol for dispersion.

The sol-gel transformation was found to be perfectly thermally reversible, thus differing from that of silicic acid, and decomposition does not appear to occur as in the case of gelatin. Dilute gels are perfectly transparent; but at higher concentrations, 0·2 % and over, an opalescence makes its appearance, increasing with the amount present in the liquid. Dilute gels, on standing,

[1] Working under a grant from the Medical Research Council.

become somewhat cloudy. On prolonged standing syneresis occurs with contraction of the gel to a core and the exudation of water. Simultaneously small nuclei make their appearance in the gel, which continue to grow to form spherites. Microscopic examination of the spherites shows them to consist of minute elongated radiating crystals. The appearance of these crystalline spherites in the gel, differing from the shot-like spheroids formed in the gelation of gelatin [Bradford, 1921], lends additional support to the two-phase theory of gel structure. The thread or fibrillar nature of the spherite group, in addition, supports the assumption that the natural crystal growth of dibenzoylcystine in water is in the form of poly-molecular crystalline rods, the gel consisting of a number of intersecting fibrils built up of these rods in which the water is enclosed. The fibrils in the gel are evidently relatively coarse, since the gel is both moist and elastic and exhibits syneresis.

The examination of the spherites thus classifies dibenzoylcystine as belonging to the fibrillary crystalline gels, which include nearly all the gels which have been examined, with the possible exception of dilute gelatin, agar and silica. These latter, although generally regarded as a class apart, may be brought into the same category, if one assumes, in the disperse phase, the existence of highly elastic or mobile fibrils undergoing a process analogous to crystal twinning.

Dibenzoylcystine is a relatively strong acid, a gel containing 2·66 g. per litre showing a p_H of 3·05 at 20°, the value being obtained both by indicators and the hydrogen electrode. This value, if the acid be considered as monobasic, corresponds to a dissociation constant of $0{\cdot}3 \times 10^{-3}$. The degree of dissociation was also determined by the conductivity method. The molecular conductivities of a 0·1 % solution and solutions more dilute were determined at 25° in a conductivity cell of the usual type, with the aid of a potentiometer, telephone and coil. The value of $\lambda \infty$ was found to be 250, a limit to which the molecular conductivity had almost attained at a dilution of 0·00014 molar. The value of K, the dissociation constant, varied from $1{\cdot}4 \times 10^{-3}$ to $1{\cdot}5 \times 10^{-3}$ with a mean value of $1{\cdot}48 \times 10^{-3}$. The acid is thus comparable to monochloroacetic acid and monobromosuccinic acid. No discontinuity in conductivity in the 0·1 % solution, which exhibits a feeble tendency to set, was observed, whether the liquid was agitated or kept at rest prior to the determination. This is a phenomenon which has been observed by McBain [1919] and his co-workers in their studies of soap solutions. The discrepancy between the dissociation constant value as calculated from the hydrogen ion concentration and determined by the conductivity method is probably to be attributed to a liquid junction potential in the former due to the non-diffusibility of the anion in the dibenzoylcystine. This discrepancy is of the order of 10 millivolts.

On the alteration of the hydrogen ion concentration of the medium by the addition of sodium hydroxide or hydrochloric acid several interesting points were noted. On the addition of increasing amounts of alkali the gel formation gradually became less, until the contents of the tube were entirely liquid.

On the addition of acid, the rate of setting increased and the gels were distinctly more opalescent in character, while on the addition of relatively large quantities of acid the gels became distinctly granular and less resistant to penetration. The gel possessed the maximum stability at the isoelectric point. The marked change in the physical characteristics on the alteration of the p_H of the solution was made evident by the following experiments.

Water Retention. One gram of dibenzoylcystine was dissolved in 6·0 cc. of hot alcohol and poured into 250 cc. of boiling water as quickly as possible with constant stirring. From this stock solution 20 cc. were run into boiling tubes which contained 10 cc. of solutions of alkali and acid of varying concentration as given in Table I. 20 cc. of the mixtures were then pipetted into Petri dishes for determinations of their setting power. The remaining 10 cc. were used for hydrogen determinations. At the end of 72 hours the jellies contained in the Petri dishes were removed and allowed to drain under their own gravity on filter papers in funnels. The liquid running through was measured. The results of these determinations are given in Table I.

Table I. 0·30 % *dibenzoylcystine.*

No.	*N*/20 H_2SO_4	*N*/10 NaOH	Water	Dibenzoyl-cystine solution	p_H 20° C.	Water drained in 2 hours from 20 cc. of mixture after setting for 3 days	Observations made on mixing and cooling
	cc.	cc.	cc	cc.		cc.	
1	0	2	8	20	3·65	16·5	Quite fluid
2	0	1	8	20	3·48	16·5	,, ,,
3	0	0·5	9·5	20	3·36	15·5	Moderately set, not very opalescent
4	0	0·25	9·75	20	3·26	13·0	Not quite stiff, pours
5	0	0	10	20	3·05	13·0	Stiff, does not pour
6	0·5	0	9·5	20	2·65	13·0	Very stiff
7	1·0	0	9·0	20	2·59	14·0	Full of air bubbles, set at once
8	1·5	0	8·5	20	2·52	13·0	,, ,, ,,
9	2·0	0	8·0	20	2·41	13·0	Opalescent, set at once
10	3·0	0	7·0	20	2·29	11·0	,, ,,
11	4·0	0	6·0	20	2·19	11·5	Very opalescent
12	8·0	0	2·0	20	1·93	11·5	Solid had separated out

It is evident as the hydrogen ion concentration decreases below p_H 3·0 the water-retaining power of the gel is very seriously diminished. In the more acid solutions a slight alteration takes place but the curve of drainage is not so regular.

In the determination of the p_H of the alkaline solutions it was noticed that the internal resistance of the cell became extremely high, possibly owing to the formation of a pellicle at the interface of the solution and the saturated potassium chloride junction fluid. It was not, however, sufficient to interfere with the readings.

Effect of Electrolytes. In common with other gels, the water-retaining power of the fibrils is much affected by the presence of neutral electrolytes. The effect of alteration of the osmotic pressure and the reduction in the free water content and of ion hydration naturally varies with the nature of the added electrolyte. Quantitative investigations on the effect of the addition of ammonium thiocyanate to a typical emulsoid albumin to gelatin and to silicon hydroxide gels are to be found in the literature.

Ammonium thiocyanate exerts a similar lyotropic effect on the dibenzoylcystine gels. Syneresis is more pronounced in the presence of small quantities of this salt and relative instability of the gel structure may be obtained in fairly dilute solutions as is indicated by the following draining experiments performed in a manner similar to those previously described.

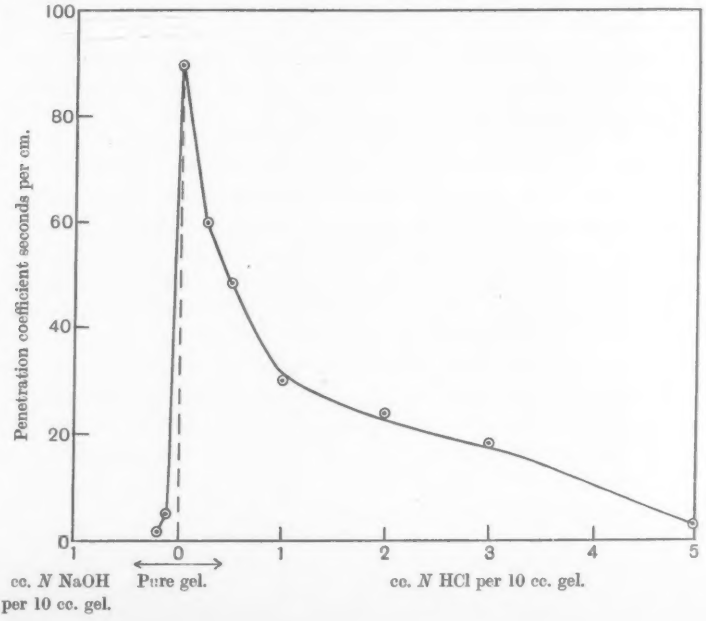
Table II. *Effect of Ammonium Thiocyanate on the setting of Dibenzoylcystine.*

0·4 % dispersion of dibenzoylcystine.
5·0 % solution of ammonium thiocyanate.

Number	Ammonium thiocyanate cc.	Water cc.	Dibenzoylcystine solution cc.	Filtrate cc. in 1 hour
1	1	9	20	14·5
2	2	8	20	17·5
3	3	7	20	18·0
4	4	6	20	19·0
5	6	4	20	21·0
6	8	2	20	21·0
7	10	0	20	21·0
8	0	10	20	14·0

Nos. 1 and 8 went solid in about 10 minutes. The others some time afterwards.

Resistance to Penetration. In order to obtain some information on the tenacity of the gels as affected by change in the p_H of the dispersion medium a simple penetration test was devised. This consisted essentially in measuring the rate of fall of a hemispherically ended plunger through a column of the gel maintained at room temperature. It was noted that a uniform velocity was attained after a fall of two or three centimetres. The time in seconds for a penetration of 1 cm. is plotted in the following curve.



It will be noticed that the influence of the alkali is extremely marked, whilst a distinct reduction in the internal viscosity is produced in relatively acid solutions. The conclusion that one may draw from these experiments is that on the addition of alkali the number of fibrils decreases, presumably because the sodium salt has no gelatinising qualities and is entirely soluble, while, on the addition of acid, the fibrils become relatively much coarser and consequently fewer in number.

Protective Action. An attempt was made to determine the protective power of dibenzoylcystine, using red colloidal gold prepared according to Zsigmondy's method. This gold gave correct values when tested against gelatin. Owing to the sparing solubility of the compound, it was impossible to carry out the test in exactly the way which Zsigmondy [1920] prescribes.

With a 0·2 % solution of the compound partial protection of 10 cc. of gold suspension occurred with 4·0 cc. of the gel. Complete protection was afforded by 6·0 cc. The gold number should therefore be about 10, which is close to that of dextrin and is nearly a negligible quantity.

Diffusion Experiments. In dilute concentrations gels such as gelatin and agar offer no abnormal resistance to the diffusion of electrolytes. With more concentrated gels the coefficient of diffusion is somewhat reduced. Attempts to estimate the fibril mesh of such gels by observations on the rate of diffusion of electrolytes, semi-colloids and colloids of varying particle diameter have frequently been made. The results, however, have been extremely variable. The same abnormalities have been noticed with dibenzoylcystine which being of much simpler chemical constitution permitted partial interpretation of the results obtained.

The experiments were made by taking a gel of 0·2 % and allowing it to set in tubes of uniform size. As soon as the gel was fairly stiff, solutions of dyes were carefully pipetted on top of it and the level marked. At intervals the entrance of the dye into the gels was estimated with calipers. All the dyes used were of Grübler's make. The results are given in Table III.

It will be noted that the gel offers very slight resistance to an electrolyte such as potassium dichromate, a distance of 100 mm. being traversed in a few hours.

Dibenzoylcystine is a negative colloid, being itself a relatively strong acid, and although the ion, as evidenced by the highly dissociated sodium salt, possesses feeble if any gelatinising properties, yet there is no doubt that the fibrils are partially ionised. In the presence of positive crystalloid or colloid dyes adsorption or mutual precipitation is to be anticipated. For such basic dyes, diffusion far into the interior is not to be looked for, and the fibrils should be markedly stained, the partition coefficient between fibril and surrounding liquid being very high. The low apparent diffusibility is for these dyes to be ascribed to adsorption and not to a small fibrillary mesh. The zone of precipitation naturally varies with the colloidal character of the dye and its diffusibility.

Table III.

Diffusion of	Nature	Adsorption	Precipitation	Diffusion rate mm. in 24 hrs.
Potassium dichromate	Crystalloid	–	–	135
Basic dyes, cationic:				
Night blue	Colloid	× ×	× ×	0
Neutral red	Semi-colloid	× × ×	×	0·2
Methyl violet 5B	,, ,,	× ×	× ×	1·0
Bismarck brown	Crystalloid	× ×	–	1·6
Toluidine blue	Colloid	× ×	×	2·0
Methylene blue	,,	×	×	2·0
Malachite green		×	–	2·0
Thionin		× ×	–	2·4
Safranin	Crystalloid	× ×	–	3·0
Fuchsin	Semi-colloid	–	–	3·0
Chemically reacting dyes:				
Eosin	Colloid	× ×	× ×	0·2
Rose bengale	,,	× ×	× ×	1·0
Acid dyes, anionic:				
Azo blue	,,	× –	–	1·1
Congo red	,,	× –	–	1·6
Benzopurpurin	,,	× –	×	1·6
Alkali blue	,,	–	–	2·0
Nigrosin		–	–	2·4
Carminic acid		–	–	3·3
Water blue		–	–	4·2
Light green F.S.		–	–	4·4
Acid fuchsin	Semi-colloid	–	–	5·0

Possible structure of the gel fibril.

Evidence is collecting to show that the union of molecules to form molecular complexes is brought about by forces identical with, though generally more feeble in character than, those uniting atom to atom within the molecule; further that these residual force fields, the product of these internal molecular asymmetries, are localised within certain definite areas in the molecule itself. These forces are electrical in character, and for convenience the various residual affinities may be designated as positive or negative relative to other such fields. The active areas on the molecules are associated with the presence of specific chemical groups which are electrically unsaturated.

The results on the diffusion of the dyes indicate that mutual precipitations of the cationic dyes and the anionic gel do actually take place, and that with the more colloidal dyes the precipitation zone is more limited and the colour more intense, confirming the work of Biltz and Teague and Buxton.

The halide-containing dyes, eosin and rose bengale, appear to react chemically with the dibenzoylcystine. But feeble, if any, adsorption was observed in the case of the anionic dyes. Those that were distinctly colloidal in character were feebly adsorbed. Benzopurpurin, a dye very similar to electrolytes, was apparently relatively easily precipitated. In general, the order of diffusibility observed with dyes and benzoylcystine was very similar to that observed by Bechold and others [1906, 1919].

Some idea of the nature of the linkages involved in the gel fibril may be obtained from a survey of the effect of substitution in dibenzoylcystine of various groups on the gel formation.

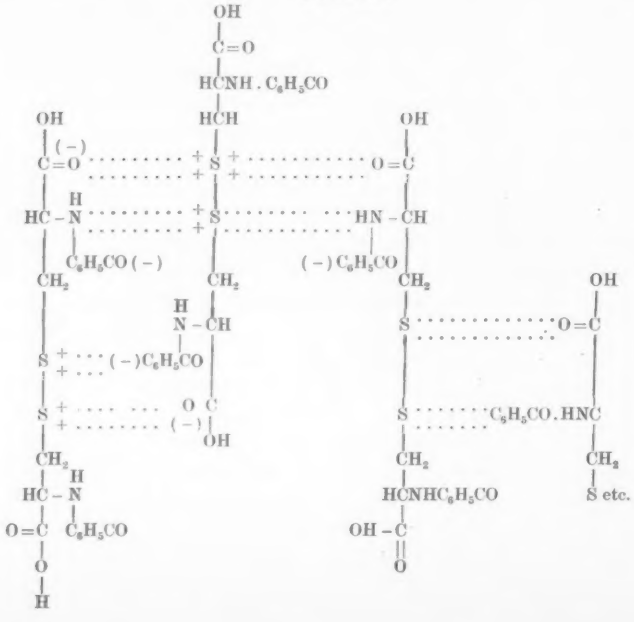
These may be:

1. Replacement of the carboxylic hydrogen by a more electropositive element. For example, Na destroys the gel-forming properties.

2. Replacement of the electronegative benzoyl group attached to the amino nitrogen by an electropositive group destroys the gel-forming power of the substance. Attempts to prepare the cinnamyl derivative, which should be more electronegative than the hydrogen or the acetyl, have not succeeded. The electronegative di-*m*-nitrobenzoylcystine has been prepared by Dr F. W. Dootson and exhibits the characteristic gelatinising qualities of the simple benzoyl compound.

3. Replacement of the —S—S— linkage by groups such as $—CH_2—CH_2—$ or —CH = CH— results in a loss of gelatinising properties. Gel formation thus appears to be dependent on the presence of an electronegative group attached to the amino nitrogen, which must not be too polar in character, on the presence of a relatively negative carboxyl group and on the presence of the electropositive —S—S— grouping. Evidence for the unsaturated and electropositive character of the —S—S— group is furnished by the interaction of cystine with halide aromatic substances in the body, *e.g.* the formation of bromophenylmercapturic acid from bromobenzene. Linkages of this double character would result in the formation of molecular aggregates arranged in echelon permitting the growth of needle-like or fibrous crystals, as shown in the following suggested formula.

Formula.



SUMMARY.

Gels of dibenzoylcystine may be prepared by peptisation with hot water. The substance is a relatively strong acid with a dissociation constant of $1{\cdot}49 \times 10^{-3}$. The gel structure appears to be fibrillary and relatively coarse. The sodium salt of the acid exhibits no gelatinising properties. The presence of acids greatly reduces the solubility of the compound. This was confirmed by observations on the water-retaining capacity and internal viscosity of the gel. Lyotropic salts, such as ammonium thiocyanate, reduce the water-retaining power of the gel, producing ultimate liquefaction. Cationic dyes are adsorbed and precipitated by the gel. Anionic dyes diffuse in a normal manner, whilst halogen dyes apparently react chemically with the sulphur group. Di-*m*-nitrobenzoylcystine has similar properties to the simple compound. A possible structure for the gel fibril based upon the effect of chemical substitution on the gel formation is suggested.

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LIII. THE NITROGEN-DISTRIBUTION IN BENCE-JONES' PROTEIN, WITH A NOTE UPON A NEW COLORIMETRIC METHOD FOR TRYPTOPHAN-ESTIMATION IN PROTEIN.

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The physical properties of Bence-Jones' protein have been more or less fully described in all investigated cases. On the other hand, there is but little reference to the exact chemical constitution and especially to the amount of individual amino-acids. Magnus-Levy [1900] determined the N-balance by Haussmann's method [1899]. Abderhalden and Rostoski [1905] estimated the monoamino-acids by esterification and Hopkins and Savory [1911] all the amino-acids by a similar method. This last investigation was carried out in two different cases and it is to one of these that the protein belongs that I have used for the analysis described in this paper. I determined the nitrogen distribution by van Slyke's method and the tryptophan content by the colorimetric estimation of von Fürth and Lieben [1920]. The details of the analysis are described later. For the clinical symptoms of this case and the physical properties of the protein the original paper by Hopkins and Savory [1911] must be consulted in which the case is described as Case C.

Table I gives the results of the different analyses carried out on Bence-Jones' protein.

Table I.

The figures are % of total nitrogen.

	Magnus-Levy	Abder-halden	Hopkins		Present analysis
			Case A	Case C	
Amide-N	8·6	.	8·02	8·00	9·43
Melanin-N	.	.	.	.	.
Total-N of bases	24·7	.	.	.	23·11
Cystine-N	.	.	.	0·41	1·25
Arginine-N	.	+	11·9	12·0	9·27
Histidine-N	.	+	1·44	1·33	4·54
Lysine-N	.	+	.	4·34	8·04
Total-N of filtrate	64·2	.	.	.	66·84
Amino-N of filtrate	.	.	.	.	61·69
Non-amino-N of filtrate	.	.	.	.	5·15
Glycine-N	.	1·95	+	+	.
Alanine-N	.	4·37	+	+	.
Valine-N	.	.	.	4·22	.
Leucine-N	.	6·98	.	4·29	.
Aspartic acid-N	.	2·89	1·34	1·41	.
Glutamic acid-N	.	3·52	4·42	4·73	.
Phenylalanine-N	.	0·78	2·47	2·57	.
Tyrosine-N	.	0·81	1·97	2·02	.
Proline-N	.	1·40	1·93	2·00	.
Oxyproline-N	.	–	–	–	.
Tryptophan-N	.	.	0·69	.	2·44

In the papers of Abderhalden [1905] and Hopkins [1911] the amounts of amino-acids are given in percentage of the protein. Assuming the total nitrogen of the protein to be 16·21 %, a value found by Hopkins, it is possible to calculate the nitrogen of each amino-acid in percentage of the total nitrogen. The above table is expressed according to the latter.

It is a matter of course that only those estimations are directly comparable which have been performed by the same method. This holds good in the two cases examined by Hopkins [1911], which show almost identical values. Moreover Hausmann's estimation of the nitrogen distribution and that by van Slyke are based on the same principle, so that those cases may also be compared. They also show a close agreement. This indicates that in the two cases examined by Hopkins [1911] and in the case of Magnus-Levy [1900] the protein shows the same relative composition of mono- and diamino-acids. The assumption that these three cases have so far the same chemical constitution by mere chance is not probable. It seems more likely that in all cases of Bence-Jones' protein-uria there appears one and the same protein or mixture of proteins in the urine. Abderhalden [1905] found in his case somewhat different values from those of Hopkins in spite of a similar method. But esterification is a very complicated method and does not give quantitative results.

Table II shows the relations between Bence-Jones' protein and those of serum and tissue.

Table II.

The figures are % of total nitrogen.

	Bence-Jones' protein	Serum-globulin[1]	Serum-albumin[2]	Normal tissues[3]	Tumors[4]
Amide-N	9·43	7·95	6·65	5·82	4·73
Melanin-N	0·90	2·30	0·95	3·09	2·42
Total-N of bases	23·11	25·25	34·75	30·71	30·54
Cystine-N	1·25	1·65	3·10	0·93	1·12
Arginine-N	9·27	8·00	9·90	11·13	10·09
Histidine-N	4·54	4·80	5·85	7·86	7·09
Lysine-N	8·04	10·80	15·90	9·83	12·04
Total-N of filtrate	66·84	64·80	58·15	60·82	62·14
Amino-N of filtrate	61·69	62·65	56·55	55·88	54·24
Non-amino-N of filtrate	5·15	2·15	1·60	4·94	7·90
N-recovered	100·28	100·50	100·30	100·4	99·8
Tryptophan-N	2·44	2·5–3·5[5]	—	—	—

[1] and [2] carried out by Hartley [1914].
[3] and [4] carried out by Drummond [1916].
[5] carried out by von Fürth and Lieben [1920].

Hartley's [1914] analysis and that described in the present paper show the same nitrogen distribution for mono- and diamino-acids in Bence-Jones' protein and serum-globulin. The serum-albumin is characterised by a higher proportion of diamino-acids. Moreover, the tryptophan content is almost identical in serum-globulin and Bence-Jones' protein. On the other hand, the latter as also the serum-albumin yields less melanin. But this fraction depends much on the hydrolysis and is therefore the most uncertain. The only real difference between serum-globulin and Bence-Jones' protein has been found in the non-amino-nitrogen of the filtrate, *i.e.* the proline and oxyproline

fraction. It is much greater in Bence-Jones' protein than in serum-globulin and approaches the amount in tissue proteins. But these, on the other hand, contain the diamino-acids in a greater proportion than Bence-Jones' protein. Analyses of the tumor-proteins in the bone-marrow have not yet been carried out. Those registered in the above table were prepared from breast-cancers. They do not show any close resemblance in their amino-acid balance with Bence-Jones' protein. But it must be understood that Drummond coagulated the proteins in tissues and tumors by heat in acid solution, which also precipitates the nucleo-proteins. It is not known in which fraction the nitrogen of the purine and pyrimidine-ring appears and in what manner the relations are changed. This may make the comparison between his analyses and those in which no nucleo-proteins are present somewhat uncertain. On the whole, Bence-Jones' protein seems to be a substance, not only characterised by its physical behaviour, but also by its distribution of amino-acids, which differs from all the proteins analysed up to the present time.

Experimental details.

A. Preparation of Bence-Jones' protein: it was precipitated by heat not exceeding 60°, washed with water, alcohol and ether, and dried at 110° till of constant weight. [See Hopkins and Savory, 1911.]

B. Analysis by van Slyke: 3–4 g. of protein were hydrolysed with a volume of 20 % HCl 35 times its weight for 48 hours at 100°. For each of the two analyses the hydrolysis was carried out separately.

The following modifications mentioned by Crowther and Raistrick [1916] were applied:

(*a*) To the solution of $N/10$ sulphuric acid were added some crystals of KI and 1–2 cc. of saturated solution of KIO_3. The iodine produced was then titrated with sodium thiosulphate using soluble starch as indicator.

(*b*) The melanin-precipitate was filtered off under slight suction.

(*c*) The amino-nitrogen was determined by the micro-apparatus. Triplicates done on 2·5 cc. varied within 2 %.

(*d*) I found it easier to estimate the total nitrogen of the bases in a different portion from arginine as Plimmer [1916] has pointed out. I carried out the arginine estimation itself in the original way described by van Slyke. Bumping was almost entirely avoided by using a rose-burner.

After concentrating the hydrolysed protein the solution was made up to 100 cc. and I proceeded in the following manner:

1. Total-N in 10 cc.: duplicate.
2. Ammonia-N in 75 cc.
3. Melanin-N in 75 cc.

The solution of the bases was made up to 100 cc. In it were estimated:

1. Total-N in 10 cc.
2. Cystine-N in 20 cc.
3. Arginine-N in 50 cc.

4. Amino-N in 2·5 cc.: triplicate.

The filtrate of the bases was made up to 200 cc. and in this were estimated:

1. Total-N in 50 cc.: duplicate.

2. Amino-N: 10 cc. were diluted to 25 cc. and 2·5 cc. taken for estimation of amino-N: triplicate.

Table III gives the results in percentage of total nitrogen of the two analyses carried out, the values corrected for solubility of the bases and according to blank tests.

Table III.

	I	II	Average
Amide-N	9·36	9·50	9·43
Melanin-N	0·92	0·82	0·90
Total-N of bases	23·05	23·18	23·11
Cystine-N	1·26	1·24	1·25
Arginine-N	9·15	9·40	9·27
Histidine-N	4·82	4·27	4·54
Lysine-N	7·82	8·27	8·04
Total-N of filtrate	66·88	66·81	66·84
Amino-N of filtrate	61·98	61·40	61·69
Non-amino-N of filtrate	4·90	5·41	5·15
Total-N recovered	100·26	100·31	100·28

C. Colorimetric estimation of tryptophan by von Fürth and Lieben. For details the original paper must be consulted [1920].

The ordinary cuvettes of the Dubosque colorimeter can be used, putting a rubber ring between the bottom and the upper part and screwing the latter tightly down. Experiments show that the fluid does not come in contact with the metal and the colour remains unchanged.

The colour reaction is better produced in two measuring flasks of 25 cc. than in two test-tubes. Thus the volume of the standard solution and that of the unknown solution can be made equal with greater accuracy.

Each time to 2 cc. of a standard solution of 0·1 % pure tryptophan (prepared in the Biochemical Laboratory of Cambridge) and to 2 cc. of a solution of Bence-Jones' protein of about 5 % were added 1 drop of formaldehyde 2½ % and 20 cc. of saturated hydrochloric acid solution. Ten minutes afterwards sodium nitrite was added, the amount varying from 10–50 drops of a 0·05 solution in order to get the maximum depth of colour.

The following estimations were carried out:

1. 4·80 % solution of Bence-Jones' protein in 0·6 % NaOH. A 20 minutes' heating at 100° dissolved the protein. 20 drops of sodium nitrite produced the maximum colour: triplicate.

2. 6·29 % solution of Bence-Jones' protein in 15 % NaOH. Dissolved in 20 minutes at 100°. 50 drops of sodium nitrite produced the maximum colour.

3. 4·57 % solution of Bence-Jones' protein in 20 % NaOH. Dissolved in 15 minutes at 100°. 50 drops of sodium nitrite produced the maximum colour.

Table IV gives the calculated tryptophan content of Bence-Jones' protein in percentage:

Table IV.

I					
a	*b*	*c*	2	3	Average
2·74	2·88	2·95	3·14	2·75	2·89

The difference between the extreme values is 14 % of the highest. The difference in the amount of alkali used in dissolving the protein and therefore the difference in the acidity of the colour solution has no influence on the colour depth. This fact ought to be noticed since von Fürth and Lieben [1920] pointed out that the colour depth in pure tryptophan solutions varies with the acidity.

In the course of the experiments I found two difficulties:

1. The standard solution often changes the character of the colour in a short time and matching becomes uncertain even with a colour-screen. This is probably due to the beginning of clouding. On the other hand, the protein solution does not cloud but reaches its maximum colour more slowly than the pure tryptophan.

2. The colour in pure tryptophan is blue, that in the protein is more or less red, so that matching without a colour-screen is impossible. It is not difficult to find a screen making the two colours identical and a solution of $CuSO_4$ proved to be best. But the following must be considered. Fearon [1920] has shown that formaldehyde and glyoxylic acid give with tryptophan two coloured compounds, which he calls tryptophan-red and tryptophan-blue respectively. The colour obtained under ordinary conditions is a mixture of both and in pure tryptophan the blue prevails, in protein the red. Since both colours are due to tryptophan it must be considered what influence a colour-screen may have on the estimation. If the screen takes away the red rays, then one part of the tryptophan molecules is eliminated and it is uncertain if the calculation only based on the remaining blue would give right values for the whole amount of tryptophan. As Fearon remarks, a reaction producing two different colours is not suitable for a colorimetric method.

The blue colour of pure tryptophan turns more red by cooling, but at 10° the colour of pure tryptophan and that of protein are not yet matchable.

There is one aldehyde which gives nearly the same blue colour in pure tryptophan and in protein solutions, namely benzaldehyde. Salicylaldehyde gives also red and blue. As to colour, benzaldehyde would therefore be the most suitable reagent for a colorimetric method. The reaction is specific for tryptophan, no other known amino-acid producing it. Pure gelatin does not give any blue colour.

I therefore tried with benzaldehyde and used the following solutions:

1. Solution of pure tryptophan of 0·05 % in distilled water.
2. Solution of pure benzaldehyde of 1·9 % in pure hydrochloric acid of about 38 %.
3. Solution of sodium nitrite of 0·05 % in distilled water.
4. Pure hydrochloric acid of about 38 %. It is most essential to use only

very pure hydrochloric acid without or only with a very slight yellow colour. Otherwise the colour reaction does not reach its maximum. On the other hand, I found it unnecessary to saturate with hydrochloric acid gas if the acid is 38 % or more.

After a good many trials the following technique proved to be best: I mixed 2 cc. of the tryptophan solution, 1 cc. of the benzaldehyde solution and 10 cc. of hydrochloric acid in a measuring flask of 25 cc. After 5 minutes I added 5 drops of the sodium nitrite solution and let it stand together for 15–60 minutes. Then I filled up to the mark with hydrochloric acid.

The experiments revealed several advantages in using benzaldehyde instead of formaldehyde.

1. The colour solution does not cloud and even after several days no precipitate is produced. There is a slight diminution of the colour depth in 24 hours, but it does not change appreciably in the course of 1 or 2 hours.

2. The maximum colour reached with benzaldehyde is about double as deep as with formaldehyde, using in both cases the same concentration of tryptophan. This is, of course, welcome in the case of more or less insoluble proteins. The solution of 0·05 % tryptophan gives a satisfactory colour depth. The reaction goes on as quickly or even more quickly with benzaldehyde.

3. Both pure tryptophan and protein give blue colours. There is a slight difference in shade and sometimes they are not matchable without a colour-screen. On page 560 I discussed the error possibly arising from this source by using formaldehyde and this holds good for benzaldehyde too. But in the latter case the two solutions are nearly the same, whereas in the former they are very different. Therefore one would assume that the error with formaldehyde might be much greater than with benzaldehyde. The estimation is uncertain in both cases with coloured protein solutions or very impure proteins. Traces of iron, for instance, hinder the colour reaction.

In order to decide if the colour depth varies proportionally to the concentration, I estimated solutions of varying concentrations of tryptophan by comparing them with a standard solution and obtained the following results:

Table V.

Number	Calculated tryptophan %	Estimated tryptophan %	Error %
1	0·1092	—	—
2	0·0546	0·0510	−6·6
3	0·0546	0·0574	+5·0
4	0·0546	0·0510	−6·6
5	0·0273	0·0256	−6·0
6	0·0546	0·0549	+0·5
7	0·0546	0·0535	−1·7
8	0·0273	0·0273	±0·0
9	0·0546	0·0549	+0·5
10	0·0546	0·0560	+2·5
11	0·0273	0·0278	+2·0
12	0·0546	0·0546	±0·0
13	0·0546	0·0568	+4·0
14	0·0273	0·0282	+5·0
Average	—	—	−0·2

There is a maximum error of 6·6 %. Von Fürth had one of 20 %, therefore benzaldehyde seems to give more accurate results. I can not give any explanation of the error. The colour reaction is capricious and an unknown influence may sometimes come into play.

I tried the same with caseinogen and got a maximum error of 5 %.

In all cases I used the same amount of benzaldehyde and of sodium nitrite. Within the above limits it is not necessary to vary them proportionally to the amount of tryptophan, since the maximum colour is reached without such a complication of the method. In comparing protein and pure tryptophan the concentration of tryptophan in both solutions ought to be about the same.

In the case of water-insoluble proteins von Fürth dissolved them in more or less alkaline solutions. Since I did the same in my estimations I had to determine the influence of alkali on the colour reaction. Instead of a neutral solution of pure tryptophan different concentrations of NaOH were used as indicated in the following table:

Table VI.

Number	% NaOH	Calculated %	Estimated %	Error %
1	0	0·1092	0·1092	—
2	10	0·1092	0·0729	−50
3	5	0·1092	0·0926	−18
4	2·5	0·1092	0·1051	−4
5	1	0·1092	0·1081	−1
6	0·5	0·1092	0·1050	−4

Up to a concentration of about 2·5 % NaOH there is practically no influence on the maximum colour. Higher concentrations diminish the colour depth.

It is essential to know that things are different in protein solutions. The following table gives the tryptophan content of Witte-Pepton dissolved in different concentrations of NaOH:

Table VII.

Number	% NaOH	Tryptophan %	Error %
1	0·6	3·0	—
2	15	2·85	−5
3	20	3·0	±0
4	30	2·9	−3

After filtering off the precipitate of NaCl the colour depth is the same in all cases. Therefore insoluble proteins may be dissolved in strong alkali of 20–30 % without changing the colour reaction. It is not sure that this holds good for all proteins and it ought to be proved in every special case by using different concentrations of alkali.

The above data show that the colour reaction is not so easily disturbed in proteins as in pure tryptophan. Therefore in making comparisons one is apt to get too high values of tryptophan in proteins.

The following table gives the tryptophan content of several proteins estimated by formaldehyde and benzaldehyde:

Table VIII.

Protein	Tryptophan-content % estimated by Benzaldehyde	Formaldehyde	Difference %
Caseinogen	1·1	1·39	27
Serum-protein	1·65	2·2	33
Ovalbumin	0·97	1·5	53
Witte-Pepton	3·0	4·8	60

In all cases formaldehyde gives values 30–60 % higher than benzaldehyde. Most probably the estimation by benzaldehyde is the more correct, since it avoids several disadvantages of the formaldehyde method. No other method for exact tryptophan estimation is known, so that a direct test is impossible. Therefore the method described in this paper seems to be the best at present available for tryptophan estimation in proteins.

SUMMARY.

1. The nitrogen distribution of Bence-Jones' protein is determined by van Slyke's method.

2. Bence-Jones' protein is a substance, not only characterised by its physical behaviour, but also by the distribution of nitrogen, which differs from that of all the proteins analysed up to the present time.

3. There is some evidence that the same protein appears in the urine in all cases of Bence-Jones' protein uria.

4. The tryptophan content of Bence-Jones' protein is estimated by von Fürth's colorimetric method. Objections to this method are discussed.

5. The writer proposes to use benzaldehyde instead of formaldehyde. There are several advantages in doing so and the method becomes more correct. The values of tryptophan estimated by formaldehyde are probably 30–60 % too high.

My very best thanks are due to Prof. F. G. Hopkins for his kind hospitality in his laboratory and his kind help and advice during the progress of this work.

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LIV. THE AUTOLYSIS OF BEEF AND MUTTON.

By WILLIAM ROBERT FEARON and DOROTHY LILIAN FOSTER.

Report to the Food Investigation Board from the Biochemical Laboratory, Cambridge.

(*Received June 13th, 1922.*)

I. THE AUTOLYSIS OF BEEF AND MUTTON COMPARED.

The study of the course of autolysis in beef and mutton described in the following experiments was undertaken in the hope that it would reveal some explanation of the inherent differences observed in the effect of cold storage on these two kinds of muscle.

It is a fact of common knowledge that beef cannot be frozen and thawed again without marked changes taking place in the appearance, palatability, and general physical state of the meat, while nothing of the sort happens when mutton is similarly treated. Frozen beef on thawing becomes bluish in colour, flabby to the touch, and, most serious of all, there is an exudation of juice which contains valuable nutrient material. "Frozen" beef must be carefully distinguished from "chilled" beef. The latter is beef which has been cooled to a temperature low enough to prevent bacterial action, but not so low as to cause the tissue fluids to become solid. Meat so treated is not immune from attack from moulds, and can only be preserved for a limited length of time.

The fundamental differences in the two kinds of flesh underlying the differences in their behaviour on freezing and subsequent thawing may be chemical or physical. Autolysis is a term used to describe a series of post-mortem chemical changes in tissues, and it was hoped that any radical difference between the chemical constitution of beef and that of mutton would reveal itself in the course of the autolysis of the two kinds of meat.

Autodigestion was the term used by early workers on this subject, and it implies that, under certain conditions, tissues digest themselves. The digestion of an organ by its own self-contained enzymes must clearly be distinguished from that produced by external agencies, as, for instance, the bacteria of putrefaction. If an excised organ is kept aseptically *in vitro*, it undergoes characteristic autolytic changes. Since certain antiseptics inhibit bacterial action, while they are without effect on enzymes, autolysis pursues a normal, or nearly normal, course *in vitro* in the presence of antiseptics such as toluene, chloroform, or acriflavine. In some pathological conditions autolysis can occur in the living animal.

As in the case of digestion by the ordinary enzymes of the alimentary canal, autodigestion is a series of processes by which the complex compounds of the tissues are broken down into simpler ones. It is certain that there are changes in the carbohydrates and fats, but the course of the protein autolysis is that which has received most study. Proteolysis implies a degradation of coagulable

protein material into simpler substances, which are not coagulated by heat, nor precipitated by certain protein reagents, and hence autolysis is accompanied by a relative increase in the non-coagulable, or soluble, nitrogen. As this is capable of easy estimation, the course of autolysis is usually followed by making successive determinations of the soluble nitrogen present.

All animal tissues are subject to post-mortem autolysis, but the process proceeds at very different rates in different organs, being most rapid in glandular tissue, such as the liver, and slowest in striated muscle. In order to develop the details of technique, a preliminary examination and comparison was made of the course of autolysis in sheep and ox liver, and essentially the same methods were later applied to the study of autolysis in muscular tissue.

Experimental details.

About one and a half pounds of the liver were transferred immediately after the death of the animal, and with as little handling as possible, to a sterile, covered dish. On arrival at the laboratory it was cut into small slices and scraped to a fine pulp with sharp scalpels on a glass plate. In this way the glandular material could be almost entirely freed from connective tissue. The pulp was well mixed, weighed in a glass dish, and made up into a 20 % suspension in toluene water. For example, in one case (Exp. 12), to exactly 400 g. of well-mixed liver pulp, 50 cc. of toluene were added and sufficient distilled water to make a total volume of 2 litres. The suspension was poured into a sterile flask with a well-fitting cork which had previously been soaked in toluene. The mixture was incubated, generally at 37°, the flasks being shaken thoroughly twice a day. The course of the autolysis was followed by successive estimations of the soluble nitrogen in an aliquot part of the whole suspension. Fractions were obtained by withdrawing a known volume by means of a blunt-ended pipette. The tip was cut off an ordinary, rather wide, 20 cc. pipette, which was then found to deliver 19·4 cc., and this was used throughout all the experiments. Provided that the suspension had been carefully made up, no difficulty was found in obtaining representative samples by this means. Samples were withdrawn immediately after making up the suspension for determination of the initial total and soluble nitrogen, and then at regular intervals, generally once a day for the first four days, and later every two or three days.

The total and soluble nitrogen were estimated by Kjeldahl's method, and the amino-nitrogen by Sörensen's formaldehyde method. The value for the total nitrogen was obtained by incinerating 20 cc. of the suspension, corresponding to a known weight of muscle, and that of the soluble nitrogen by incinerating an aliquot part of the filtrate after precipitating the coagulable protein. In all cases the ammonia was steam-distilled into the standard acid, which was N for the total, and $N/10$ for the soluble nitrogen. The caustic soda was used in equivalent strengths, and was CO_2 free. The indicator used was methyl red.

The coagulable protein was precipitated either by 25 % metaphosphoric acid, or 2·5 % trichloroacetic acid, according as the filtrate was to be used for the determination of amino-nitrogen or not. It was found impossible to do a formaldehyde titration in the presence of so large an excess of phosphate, while trichloroacetic acid showed no such "buffer" action.

The following is a detailed account of the procedure. 20 cc. of the suspension were run into a stoppered graduated cylinder, and 10 cc. of a 25 % solution of glacial phosphoric acid (freshly made) added, and the volume made up to 100 cc. After thorough shaking, the cylinder was allowed to stand over night. The precipitate was then removed by filtration through a dry, fluted paper, when a crystal clear filtrate was obtained. The nitrogen was estimated in 10 cc. of this filtrate. Duplicate estimations were always made. When trichloroacetic acid was used, 50 cc. of a 2·5 % solution were added instead of the phosphoric acid, but the procedure was otherwise the same.

Expression of results.

In reviewing the literature on this subject, a great diversity in the method of expressing results has been found. The most general practice has been to give the number of cc. of acid neutralised in the Kjeldahl estimation of the nitrogen; sometimes the amount of alkali equivalent to the excess of acid. As each observer chooses his own standards, this method is far from satisfactory, and it is suggested that a uniform method of expressing results should be adopted. We have found that the clearest picture of the course of autolysis is presented by expressing the amount of non-coagulable nitrogen present as a percentage of the original total nitrogen. In this way the progressive degradation of the nitrogenous complexes is most easily followed and graphically represented.

THE AUTOLYSIS OF OX AND SHEEP LIVER.

It was found that the autolysis of the livers of both these animals follows a normal course, and they are strictly comparable. There is nothing in the breakdown of the chemical complexes after death to suggest any fundamental difference during life. The curves representing the increase in soluble nitrogen follow the same course and are similar to those obtained by other workers. Estimations were not made sufficiently soon after death to show the latent period, but both show a rapid initial increase followed after above five days by equilibrium. The curves obtained from amino-acid titrations show the same characteristics. Curves from a typical experiment are appended (Figs. 1 and 2).

THE AUTOLYSIS OF SHEEP AND OX MUSCLE.

In the main the methods used for the examination of the autolysis of liver were applicable to muscle tissue, but slight modifications were necessary in preparing the suspension. The meat was separated from fat and connective tissue, and then shredded on a glass plate. The resulting pulp was passed

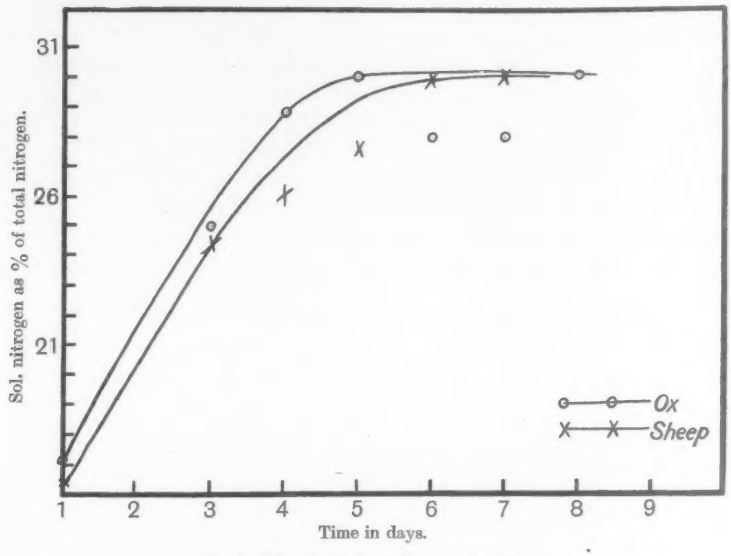

Fig. 1. The autolysis of sheep and ox liver.

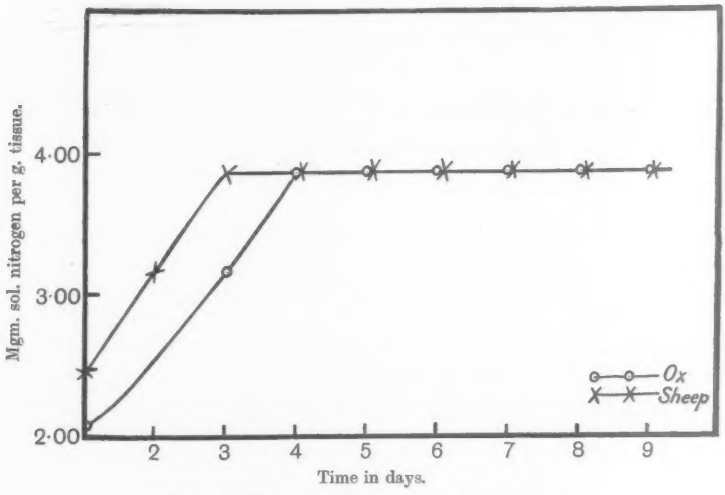

Fig. 2. The amino-nitrogen curve of liver autolysis.

twice through a fine mincer, and then rubbed to a paste in a mortar. A definite amount (*e.g.* 100 g.) was then weighed on a tared clock glass and transferred quantitatively to the mortar, and well mixed with water and toluene. The suspension was washed into a cylinder, and made up to a known volume; a fixed volume of water was then used to wash this into a sterile flask, so that the total volume of suspension was accurately known. The flask was closed by a cork soaked in toluene. In general, 20 % solutions in 5 % toluene were used, and 20 cc. fractions withdrawn as already described. The technique of precipitation etc. was exactly the same as for liver autolysis.

As in the case of the liver, the study of post-mortem autolysis in these two species of muscle failed to reveal any intrinsic differences. It was found,

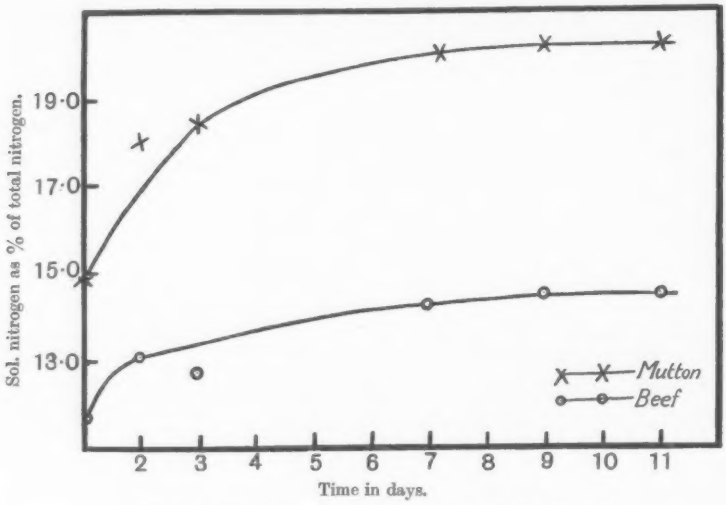

Fig. 3. The autolysis of beef and mutton at 37°. (Exp. 13.)

however, that in mutton the soluble nitrogen forms a larger percentage of the total nitrogen, so that the curves are parallel, but not superposable. The course of both, however, is perfectly regular and typical, and there is nothing to point to any important differences in the chemical constitution of these two kinds of muscle sufficient to account for their different behaviour in the cold store.

The progress of the autolysis is markedly slower in muscle than in glandular tissue, and equilibrium is not reached till about the eighth day. Moreover, the degradation stops short at a smaller percentage of soluble nitrogen.

Curves are given of a typical autolysis at 37°, and also of one at a much lower temperature. It is to be noted that though autolysis is retarded at the lower temperature, the two curves remain parallel (Figs. 3, 4 and 5).

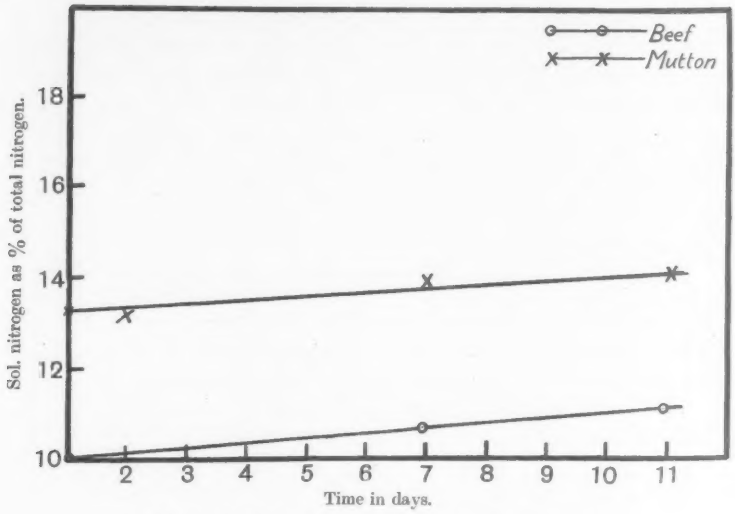

Fig. 4. Autolysis of beef and mutton at 6°. (Exp. 13.)

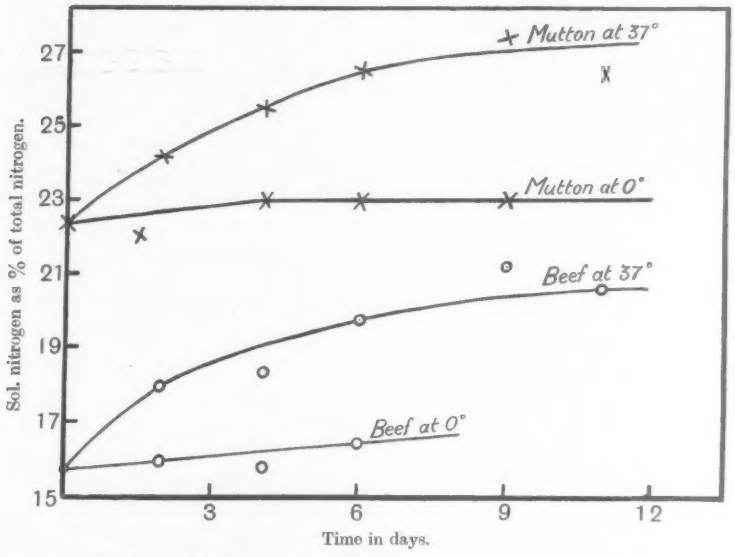

Fig. 5. The autolysis of beef and mutton at 37° and at 0°. (Exp. 14.)

General conclusions and summary.

An examination of the post-mortem autolysis of beef and mutton has thrown no light on the cause of their different behaviour after being frozen. The processes in both are exactly parallel, both at incubator temperature and at low temperatures. In the case of mutton, equilibrium is reached at a higher percentage of soluble nitrogen than in beef; but the initial non-coagulable nitrogen is higher, so that the curves are comparable. It is probable, therefore, that the differences in the two kinds of muscle in this respect lie not in their chemical constitution, but in the structure and physical properties of the fibres.

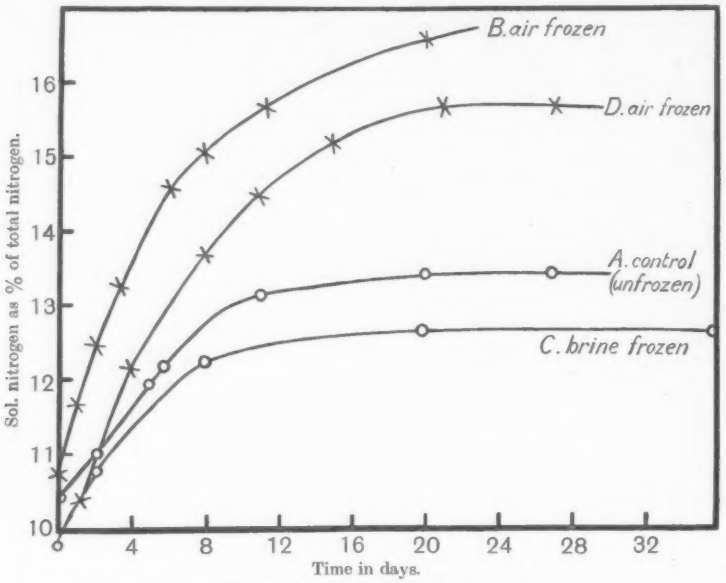

Fig. 6. The autolysis of frozen and unfrozen beef.

II. THE AUTOLYSIS OF FROZEN BEEF.

While working on the autolysis of beef it seemed of interest to determine whether previous freezing had any influence on the course of autolysis after thawing. It was found that not only was the course and degree of autolysis profoundly altered by such treatment, but that the method of freezing, that is the rate of freezing, is of great significance. This is made clear by the curves given in Fig. 6. It will be seen that previous freezing of the muscle results in a much greater degradation of the nitrogenous constituents during autolysis, and also that equilibrium is greatly delayed. The rate of freezing of carcasses is known commercially to be most important, and recent work has shown that, if frozen sufficiently rapidly, the meat on thawing approaches

more nearly in appearance and palatability to fresh beef. On the large scale, rapid cooling is achieved by immersing the carcass in brine at about – 20° C. instead of freezing in air chambers. The beneficial effect of rapid freezing is borne out by the autolysis curve of beef frozen in brine. This curve approaches much more closely to that of the control from fresh beef, than that of air-frozen meat.

The following are the details of one such experiment. The animal was killed at 2.30 p.m. on June 29th, and immediately, before skinning, about 2½ lbs. were cut off the shin, and placed in a sterile pan in ice. On arrival at the laboratory, the skin and fat were removed, and the muscle divided into four portions.

"A" was used as control, and a 20 % suspension was made by the method described above, and placed in the incubator at once.

"B" was suspended in a glass jar, and placed in the cold store at 18° F. at 3.20 p.m. It was removed the next day, and allowed to thaw for six hours at room temperature, and then the autolysis suspension made up as "A."

"C" was treated in the same way as "B" except that, in the cold store, it was sunk in saturated brine at 18° F.

"D" was kept at 0° for 20 hours, and then placed in cold store on June 30th, after which it received the same treatment as "B."

In each case the autolytic mixtures consisted of 100 g. muscle in 5 % toluene. Samples were removed at the times indicated on the curve, and the subsequent procedure was exactly as previously described.

Curves are given for one experiment only, but similar relationships were found to hold in other experiments.

It is very difficult to explain this effect on autolysis produced by freezing. Obviously the equilibria of the cell have been disturbed by the rupture of the membranes which occurs during slow freezing. As there is an absence of "drip" after brine freezing it would seem that by this method any extensive damage to the cell is avoided, and this is borne out by the autolytic changes in the brine-frozen beef.

Our sincere thanks are due to Prof. Hopkins for his interest and help throughout this work.

LV. NOTE ON THE OXIDATION OF CARBOHYDRATES WITH NITRIC ACID.

By PAUL HAAS and BARBARA RUSSELL-WELLS.

(*Received June 16th, 1922.*)

During the course of an examination of the carbohydrate constituent of *Chondrus crispus* we had occasion to oxidise the material with nitric acid, with a view to determining the amount of mucic acid produced. On examining the mother liquor from the mucic acid it was found to have a powerful reducing action upon Fehling's solution in the cold. On repeating the oxidation under rather more drastic conditions and evaporating the mixture to a syrup the same result was obtained. This seemed sufficiently remarkable to be worth investigating and samples of cane sugar, lactose, glucose and levulose were accordingly oxidised in the same way, and in every case it was found that the resulting product readily reduced Fehling's solution in the cold. Experiments were thereupon started upon pure glucose with the intention of isolating the material responsible for this strong reducing action. While these experiments were in progress a paper appeared by Kiliani [1922] in which he described the production of certain new reducing acids formed by carefully oxidising various carbohydrates at room temperature in the absence of air. As this author expressed the wish to reserve this field for himself we felt bound to discontinue our investigation, but thought it desirable to put on record our observations so far as they go in order to draw attention to the fact that powerful reducing substances are produced, not only under the rather peculiar conditions described by Kiliani, but even by oxidation of carbohydrates with hot nitric acid and without special precautions, although the yields obtained may possibly be smaller than those obtained by Kiliani. 25 g. of glucose were dissolved in 100 cc. of nitric acid (sp. gr. 1·15) and warmed over a boiling water-bath; after a short time a violent action set in which gradually subsided and the heating was continued until the solution had been reduced to one-third of its original volume. In order to avoid undue dilution the solution was neutralised by the addition of powdered crystallised sodium carbonate. The resulting liquid, which was of a light yellow colour, was found to darken slowly even when kept in a stoppered bottle protected from the light; it reduced Fehling's solution at once in the cold and likewise ammoniacal silver oxide.

The solution was treated with 20 % lead nitrate until no further precipitate was formed. This precipitate, *A*, was filtered off and when washed, suspended in water and decomposed with hydrogen sulphide yielded a solution which reduced ammoniacal silver nitrate but not Fehling's solution. The filtrate from precipitate *A*, though containing an excess of lead nitrate, gave no further precipitate on standing; the fact that the solution still reduced Fehling's

solution at once in the cold was taken to indicate that the reducing substance did not form an insoluble lead salt and was possibly not an acid, but on investigation it was found that the solution had acquired a markedly acid reaction, and on addition of a little alkali until the reaction was just on the acid side of neutrality a further heavy precipitate *B* was formed, which, after washing and decomposing with hydrogen sulphide, yielded an acid which reduced ammoniacal silver nitrate and Fehling's solution on heating but not in the cold. The filtrate from precipitate *B* which still reduced Fehling's solution in the cold was next treated with basic lead acetate, when it yielded a third precipitate *C*; the latter, when decomposed, gave the unknown acid *X* for whose isolation these experiments were undertaken; it was found to reduce both ammoniacal silver nitrate and Fehling's solution in the cold. The filtrate from precipitate *C* no longer had any reducing properties.

Subsequently it was found that the same acid *X* could be more rapidly obtained by replacing lead nitrate by lead acetate, since in using this reagent there was no change in the reaction of the solution; the addition of an excess of lead acetate caused the simultaneous precipitation of both the precipitates *A* and *B* of the previous experiment, leaving a filtrate containing only the acid *X* which could then be precipitated by the addition of basic lead acetate; after adding an excess of this reagent the precipitate was filtered and washed repeatedly with hot water until the washings were free from nitrate, an operation which resulted in considerable loss owing to the tendency of the precipitate to go through the filter paper. The washed precipitate decomposed with hydrogen sulphide yielded a powerfully reducing acid solution which, evaporated *in vacuo*, left a pale brown, viscous material which would not crystallise and which gradually darkened on keeping, especially if heated. This substance, dissolved in water and heated with concentrated hydrochloric acid, evolved a considerable quantity of furfural, thus showing it to contain at least five carbon atoms. The aqueous acid solution, treated with baryta solution, precipitated a small quantity of a sparingly soluble salt, the filtrate from which, however, still contained barium and reduced Fehling's solution in the cold. The neutral solution now also gave an immediate precipitate with lead acetate although this reagent would not precipitate it from the original oxidation mixture; this precipitate dissolved in caustic soda still reduced Fehling's solution in the cold.

Attempts to prepare a crystalline oxime, phenylhydrazone, *p*-bromophenylhydrazone and a cinchonine salt were unsuccessful. From the above facts it would appear that this substance, though in some respects similar to glycuronic acid, is not identical with it, but in view of the circumstances set forth above (with regard to the claim by Kiliani to reserve the field) and the possible identity of this substance with one of the compounds obtained by this author we have decided not to pursue the investigation any further.

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LVI. OPSONINS AND DIETS DEFICIENT IN VITAMINS.

By GEORGE MARSHALL FINDLAY and RONALD MACKENZIE.

From the Royal College of Physicians' Laboratory, Edinburgh.

(*Received June 19th, 1922.*)

Although it is well known that animals fed on diets deficient in vitamins frequently die from intercurrent bacterial infections, the reason for this apparent failure in the protective mechanism of the body is at present unknown. Hektoen [1914] found that the serum reactions were quite normal in animals fed for considerable periods on artificial diets, while later, Zilva [1919] could not determine any decrease in the power to form agglutinins, complement and immune body in animals fed on diets deficient in vitamins.

In the present investigation the opsonic activities of the serum were studied in animals fed on diets deficient in vitamins.

Diets deficient in vitamin *A*.

Rats were employed for this experiment. Four partially grown rats varying from 106 to 120 g. were placed on a diet consisting of caseinogen, starch, cotton seed oil, marmite and an inorganic salt mixture. After ten weeks the animals were killed. One of the animals was suffering from keratomalacia at the time of its death. The sera from the four animals were collected in the usual manner and pooled before use.

Four normal animals fed on a diet of caseinogen, starch, cod-liver oil, marmite and inorganic salts were also killed and their sera collected to serve as a control. Leucocytes were obtained from a normal rat. The technique employed was that recommended by Wright [1921].

The opsonic activity of the two groups of sera was determined for two types of organisms, *Bacillus coli* and *Staphylococcus aureus*. One hundred polymorphonuclear leucocytes were counted on each slide. Notes were taken of (1) the number of leucocytes containing bacteria, (2) the total number of bacteria phagocytosed. The average number of bacteria per polymorphonuclear leucocyte was termed the phagocytic index. The data are recorded in Table I.

Table I.

Date of examination	Organism	Condition of animal	No. of polymorphonuclear leucocytes counted	No. of cells with bacteria	Total no. of organisms ingested	Phagocytic index
After 10 weeks on the diet	Staphylococcus	− Vitamin *A*	100	79	128	1·3
	B. coli		100	40	46	0·5
	Staphylococcus	+ Vitamin *A*	100	84	136	1·4
	B. coli		100	42	69	0·7

It will be seen that there is no significant difference in the readings obtained with the two sera and thus little evidence of any decrease in the opsonic activity as the result of a diet deficient in vitamin *A*.

Diets deficient in vitamin *B*.

Rats were also used for this experiment. Six rats were fed on a diet of caseinogen, starch, cod-liver oil and an inorganic salt mixture. Three rats were killed after 21 days, the remainder after 6 weeks on the diet. Rats fed on a complete diet were used as controls. The technique employed was the same as in the first experiment. The data are given in Table II.

Table II.

Date of examination	Organism	Condition of animal	No. of polymorphonuclear leucocytes counted	No. of cells with bacteria	Total no. of organisms ingested	Phagocytic index
After 3 weeks on the diet	Staphylococcus	− Vitamin *B*	100	73	96	1·0
	B. coli		100	74	98	1·0
	Staphylococcus	+ Vitamin *B*	100	70	129	1·3
	B. coli		100	82	126	1·3
After 6 weeks on the diet	Staphylococcus	− Vitamin *B*	100	77	135	1·3
	B. coli		100	59	79	0·8
	Staphylococcus	+ Vitamin *B*	100	67	141	1·4
	B. coli		100	76	88	0·9

Diets deficient in vitamin *C*.

Guinea-pigs were used for this experiment. As is well known, when fed on a diet containing only small quantities of vitamin *C* these animals develop chronic scurvy, a condition characterised by no very definite clinical symptoms except a failure of normal growth. Six guinea-pigs, varying in weight from 250 to 300 g., were fed on an *ad libitum* diet of oats and bran, and 70 cc. of autoclaved milk a day, with the addition of 2 cc. of orange juice every third day. Six controls were placed on the same basal diet but with the addition of 10 cc. of orange juice every day. Experiments, as before, were carried out with two organisms, *Staphylococcus aureus* and *Bacillus coli*. Readings were made after

four weeks and after eight weeks on the dietary, three scorbutic and three control animals being killed on each occasion. The technique was the same as in the experiments with rats except that the leucocytes in this instance were those of a normal guinea-pig. The results are summarised in Table III.

Table III.

Date of examination	Organism	Condition of animal	No. of polymorphonuclear leucocytes counted	No. of cells with bacteria	Total no. of organisms ingested	Phagocytic index
After 4 weeks on the diet	Staphylococcus	− Vitamin *C*	100	94	315	3·1
	B. coli		100	87	115	1·1
	Staphylococcus	+ Vitamin *C*	100	91	330	3·3
	B. coli		100	84	148	1·5
After 8 weeks on the diet	Staphylococcus	− Vitamin *C*	100	87	261	2·6
	B. coli		100	82	104	1·0
	Staphylococcus	+ Vitamin *C*	100	92	282	2·8
	B. coli		100	76	95	0·9

THE PHAGOCYTIC ACTIVITY OF THE POLYMORPHONUCLEAR LEUCOCYTES IN SCURVY.

Since the opsonic activity of the serum was practically the same both in scorbutic and in control animals, an attempt was next made to determine whether the liability to infection in scurvy was in any way associated with a decreased phagocytic power of the polymorphonuclear leucocytes themselves. For this purpose two guinea-pigs of equal weight were selected, one being quite healthy, while the other had been fed on a diet deficient in vitamin *C* for the previous eight weeks. 1 cc. of a suspension of *S. aureus* heated for an hour at 55° and containing 200 million organisms was injected intraperitoneally into each animal. At intervals some of the peritoneal exudate was removed by means of a capillary tube; smears were made, stained with "Leishman" and examined microscopically. Two hundred polymorphonuclear leucocytes were counted on each film, a note being taken of the number of polymorphonuclear leucocytes containing 0, 1, 2, 3, and more than 3 staphylococci. The results are shown in Table IV.

Table IV.

		Percentage no. of polymorphonuclear leucocytes containing				
Time	Condition of animal	No Staphylococci	1 Staphylococcus	2 Staphylococci	3 Staphylococci	More than 3 Staphylococci
3 hours after injection	Scorbutic	50	27	13	8	2
	Control	44	26	16	10	4
6 hours after injection	Scorbutic	16	32	22	16	14
	Control	33	18	25	12	12
18 hours after injection	Scorbutic	36	24	26	6	8
	Control	36	18	24	16	6
30 hours after injection	Scorbutic	48	16	21	9	6
	Control	37	20	21	13	9

An examination of the pleural exudate 54 hours after the injection showed that mononuclear leucocytes were now the predominant type of cell in both animals while the polymorphonuclear leucocytes still present were undergoing degeneration.

There is no evidence to suggest any decrease in the phagocytic activity of the polymorphonuclear leucocytes as the result of a diet deficient in vitamin *C*.

In many ways the bacterial infections so frequently found in association with the deficiency diseases are analogous to the terminal infections met with in other chronic diseases. It is therefore of some interest to note that quite recently Cross [1921] has investigated the question of the opsonic index in relation to terminal infections associated with chronic bacterial infections. He was unable to determine any decrease in activity against any bacteria not concerned in the primary infection even in the very last stages of the disease.

As Bordet [1909] was the first to point out, the phagocytic power of the body would appear to be a relatively stable function and one not easily influenced by conditions which profoundly affect other vital activities.

Conclusions.

1. Rats fed on diets deficient in vitamins *A* and *B* do not show any decrease in the opsonic activity of the serum.

2. Guinea-pigs fed on a diet deficient in vitamin *C* do not show any decrease in the opsonic activity of the serum.

3. The phagocytic activity of the polymorphonuclear leucocytes of guinea-pigs with chronic scurvy is not decreased.

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LVII. ON CARRAGEEN (*CHONDRUS CRISPUS*). III. THE CONSTITUTION OF THE CELL WALL[1].

By BARBARA RUSSELL-WELLS.

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Historical.

Many workers have from time to time investigated *Chondrus crispus*, but of recent years the most exhaustive work has been done by Sebor. The presence of galactose had been established by Flückiger and Obermayer [1868], and Bente [1875, 1876] had found among the products of the action of acid upon carrageen levulinic acid and a sugar, which showed little or no optical activity. Haedicke, Bauer and Tollens [1867] established in carrageen mucilage the presence of fructose and calculated that it contained 28 % of galactose. They suggested that these sugars might be present under the form of raffinose. Sebor [1900] determined to identify the remaining sugars. He confirmed the presence of galactose and fructose, found small quantities of pentosan and showed that raffinose is not present. He also stated that he had found evidence of glucose. He oxidised the carrageen mucilage, but, after filtering off the mucic acid, failed to obtain any acid potassium saccharate on treating with potassium carbonate and acetic acid. As an analysis of the silver salt gives the same result for both mucate and saccharate he tested the potassium solution for optical activity, but found it inactive, whereas the saccharate should be active. He therefore abandoned the oxidation products and tested for glucose in a hydrolysed solution of mucilage. He hydrolysed with 6*N* HCl to destroy the fructose, and when the solution no longer gave a Selivanoff reaction, added benzylphenylhydrazine. He separated off the benzylphenylhydrazone of galactose and found the remaining compound had a melting point of 163°, that of glucose benzylphenylhydrazone being 165°. He declared that the hydrazone was a mixture and his paper gives the impression that he was not satisfied with the agreement of melting points referred to above. This appears to be all the evidence he had in favour of the presence of glucose among the constituents of carrageen. Müther and Tollens [1904] state that glucose is among the constituents of carrageen, but all the evidence they offer is an estimation of the amount of silver present in the silver salt of an oxidation product; this is obviously useless since the amount of silver is the same in the salts of both saccharic and mucic acids.

Abderhalden's *Biochemisches Handlexikon* [1911] contains the statement that the pure mucilage of carrageen corresponds to the formula $C_6H_{10}O_5$, but

[1] Thesis approved for the Degree of Doctor of Philosophy in the University of London.

it is not stated on what evidence this formula is based; it must be borne in mind that the mucilage is known to contain both nitrogen and considerable quantities of ash. Stocks and White [1903], on the other hand, have shown by analysis that the mucilage does not possess this formula.

	Found for ash free substance	Calculated for $C_6H_{10}O_5$
Carbon	37·94 %	44·44 %
Hydrogen	5·92	6·17
Oxygen	54·95	49·39
Nitrogen	1·19	—

These figures conclusively demonstrate that the formula $C_6H_{10}O_5$ does not agree with the results of analysis.

Since, as will appear later, it has now been proved that carrageen mucilage contains at least two substances which are carbohydrate esters of sulphuric acid, and various metallic radicles, any calculations of the above nature are futile.

Previous investigators have commented on the high ash content of carrageen and reported that it is largely composed of calcium sulphate, but most of them have ignored the significance of this and proceeded to examine the organic radicles obtained by various means from the plant. They also appear to have worked either on the seaweed itself or on a direct hot water extract. In 1921 Haas and T. G. Hill [1921] showed that this extract consists in reality of two distinct fractions, and in the same year Haas [1921] published a method for separating the two, together with a systematic investigation of the inorganic constituents of one of them. He found that while one was readily soluble in both hot and cold water, the other was readily soluble in hot, but only sparingly in cold water. The method of extraction and separation was based on this difference of solubility, and was, with slight modification, the method adopted in the preparation of larger quantities of the two substances required for the present investigation.

The distinctions between the hot extract (H.E.) and the cold extract (C.E.) described by Haas and Hill were confined to physical characteristics such as different solubility in cold water and different gelatinising properties.

It was also shown by Haas that the H.E. contained 17·6 % of ash which could not be reduced by dialysis and was found to be due to the presence of the calcium salt of an ethereal sulphate, in which the calcium is fully ionised and can be precipitated by the ordinary reagents, while the sulphate radicle is not ionised and can only be precipitated by barium chloride after hydrolysis of the compound.

Haas and Hill also state that they found nitrogen in both fractions. They also got positive reactions for protein in the seaweed itself and in the water-soluble product obtained from it.

From the foregoing it will be seen that carrageen can be separated into three constituent fractions, the cold water and hot water soluble portions and the residue. The physical characteristics of thetwo extracts have been described

by these previous workers, and the presence of an ethereal sulphate complex definitely established in the H.E.

Present investigation.

The present investigation was undertaken with a view to instituting a chemical comparison between the two extracts of carrageen, both as to their inorganic and organic constituents, to examine the residue of the weed, remaining after the extraction of both fractions, for cellulose and to discover if other examples could be found among the seaweeds of ethereal sulphate grouping.

The hand picked carrageen was first washed free from dust by holding it for a few seconds in running water, and then soaked for about an hour in distilled water. The aqueous extract thus obtained was poured off, filtered and evaporated, the resulting scales constituting the "cold extract." This process was then repeated and thus a further quantity of C.E. obtained. After only two hours' soaking much C.E. still remained in the weed but it was decided to sacrifice this quantity lest a more prolonged extraction should result in contamination with some of the H.E. which is also slightly soluble in cold water. The weed was therefore washed for several days by soaking in water until it was no longer slimy, all the wash water being rejected. The weed thus deprived of its C.E and some of its H.E. was then washed several times in distilled water and air-dried. In order to obtain the H.E. the dried carrageen was heated on a water-bath with fresh distilled water and the liquid from this yielded, on filtration and evaporation, the "hot extract."

Examination of the cold extract.

The crude C.E., just as it is taken from the evaporating pans, has a distinctly saline taste and on incineration was found to contain about 32 % of ash which showed a marked tendency to fuse. After dialysis the salt taste had disappeared and the ash content had fallen to 21·86 %. No amount of dialysis would reduce the ash further. The salts which dialysed out were, no doubt, mainly impurities arising from contact of the seaweed with sea-water. During dialysis it was observed that the water outside the dialyser turned slightly yellow as though some organic substance had come through the parchment. This water was therefore collected and evaporated down over a water-bath on tin-lined copper trays. A small quantity of a brown, sticky, amorphous material was obtained; this did not dry in scales like the hot and cold extracts, and although composed very largely of the salts which had dialysed out from the C.E., gave a strong reaction with Molisch's reagent, thus showing the presence of a diffusible carbohydrate; as it did not reduce Fehling's solution to any appreciable extent it was thought possible that it might be trehalose, which Kylin [1915] claims to have found in carra-

geen. Accordingly a dilute solution was tested in a polarimeter, but not the slightest trace of rotation could be observed.

A qualitative analysis of the ash of the C.E. showed the presence of sulphate, calcium, magnesium and small quantities of sodium and potassium with traces of iron. These were all found in the ash of the H.E. as well, but here the amount of alkali metals was so small that the ash showed no tendency to fuse during the process of incineration.

The calcium in the C.E. as in the H.E. is fully ionised and can be precipitated quantitatively from a 0·35 % solution by means of ammonium oxalate. The actual figures obtained were as follow:

1. Calcium by incineration 3·98 %.
2. Calcium by direct precipitation 4·03 %.

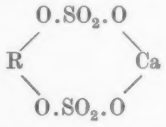
The sulphate however is not ionised, no precipitate being formed by the addition of barium chloride. But, after hydrolysis with hydrochloric acid, barium chloride gives a copious precipitate of barium sulphate, showing that hydrolysis liberates the sulphate radicle in an ionised condition, while previously it was bound to some organic radicle. The method of hydrolysis employed was as follows:

About 0·5 g. of C.E. was dissolved in 200 cc. of water and 5 cc. of concentrated hydrochloric acid added. This solution was boiled over a Bunsen flame for at least six hours to ensure complete hydrolysis. On estimation the percentage of sulphate obtained after hydrolysis was found to be rather more than twice that obtained on incineration, the actual figures being:

	I	II	Mean
1. SO_4 by incineration	13·75 %	14·16 %	13·95 %
2. SO_4 by hydrolysis	30·14	30·32	30·23

As in the case of the H.E. it is extremely difficult to get rid of all moisture, the substance tending to char when heated in a steam oven owing to the liberation of sulphuric acid.

If the C.E. contained only a calcium salt of the ethereal sulphate corresponding to the formula which has been given for H.E., viz.



the sulphate ratio would be exactly two to one. Actually, however, it is more than two to one. This discrepancy could be accounted for on the assumption that some of the ethereal sulphate was combined with ammonium instead of with calcium. Such a combination might be present in a mixed calcium and ammonium salt, or there might be slight traces of the pure ammonium salt mixed with the calcium salt. There is not, however, enough evidence to show which combination is the more probable. It is obvious that in a salt of this

nature the sulphate moiety combined with (NH_4) would leave no ash on incineration, although such sulphate could be estimated after hydrolysis.

In order to discover if any ammonium radicle were in fact present, some of the C.E. was distilled with magnesium oxide, when ammonia was given off. This ammonia was estimated and the results, calculated as N, were as follow:

	I	II	Mean
N by magnesium oxide	0·24 %	0·22 %	0·23 %

The quantity of nitrogen found would, in the form of ammonium, satisfy 0·80 % of sulphate calculated as SO_4. Thus assuming the nitrogen actually present is contained in such an ammonium salt as suggested above, the experimental value for incinerated sulphate is raised by 0·80 % from 13·95 % to 14·75 %, which is only 0·36 % below the value required by theory, a difference well within the limits of experimental error. In addition to the nitrogen which can be driven off by distillation with magnesium oxide, C.E. contains other nitrogen not liberated in this manner.

	I	II
N by Kjeldahl	0·55 %	0·57 %

As shown above, nearly half this nitrogen can be liberated by boiling with magnesium oxide and is therefore probably present in an ammonium radicle. The rest is almost certainly there as protein nitrogen. That protein is present in both H.E. and C.E. is indicated by the fact that both give a positive reaction with Millon's reagent and with nitric acid. Whether this protein is combined or not is at present impossible to say, but it is constantly present though it may be removed by prolonged heating with alkali.

Ammonium salt of extracts.

An attempt was made to prepare the ammonium salt of the C.E. in the hope of removing all the calcium and magnesium and so obtaining an ash free material. For this purpose some C.E. was dissolved in water, ammonia and ammonium chloride were added and then enough ammonium phosphate to precipitate all the calcium and magnesium. The solution was left to cool and settle over night and was filtered next morning. The liquid was filtered with the aid of the filter pump first through a Chardin and then through a quantitative filter-paper on a Buchner funnel. The filtrate, which was much clearer than a solution of ordinary C.E., was dialysed for five days to remove excess of ammonium phosphate and other impurities. Since it charred when evaporated to dryness over a water-bath, the filtrate was evaporated in a vacuum-desiccator and clear, almost colourless scales were obtained. These were dried to constant weight in the desiccator, and it was then found that the ash content had fallen from 21·26 % to 5·87 %. A qualitative analysis of the ash showed it to consist very largely of magnesia, from which it was concluded that the magnesium present in the C.E. is not in an ionised condition, or else it would have been precipitated by the phosphate.

In addition to magnesium the ash of the ammonium salt of the C.E. contained potassium and sodium but no calcium.

The same procedure was adopted with the H.E. The filtrate, after precipitation with ammonium phosphate, was dialysed for six days, and then evaporated over a water-bath in a platinum basin. Like the C.E. it showed a tendency to char, and when evaporated to small bulk turned acid and reduced Fehling's solution. It was therefore kept alkaline with ammonia all the time it was being evaporated, and when reduced to a small volume it was taken from the water-bath and set aside to cool. After cooling it set to a stiff jelly, proving that the loss of calcium does not destroy the gelatinising properties of the H.E. On complete evaporation in a vacuum the substance was found to be very like the ammonium salt of the C.E. in appearance, and consisted of clear, nearly colourless scales which readily absorbed moisture from the atmosphere. On incineration a considerable amount of ash was left, which like that of the C.E. contained magnesium. So here again the magnesium is present in an un-ionised condition.

Another sample of the ammonium salt of the H.E. was prepared by precipitating with ammonium oxalate instead of with ammonium phosphate and the ash in this was estimated and found to have been reduced from the original 17·6 % to 4·38 %.

Comparison of organic substances in hot and cold extract.

The organic complexes in the two extracts of carrageen were also investigated with a view to discovering further differences of composition between the two fractions.

Both H.E. and C.E. consist largely of carbohydrates. Neither of them will reduce Fehling's solution without previous hydrolysis, but both will do so after such treatment.

Since galactose has been found in carrageen mucilage by Flückiger and Obermayer [1868] and in *Chondrus elatus* by Takahashi [1920] it was decided to test for it in the two extracts. They were therefore oxidised with nitric acid according to the Creydt modification of the Kent and Tollens method. Mucic acid was obtained from both, thus proving galactose to be present in both fractions. The amount of mucic acid obtained was then estimated, according to the method of van der Haar [1920], the average values obtained being 21·12 % from the H.E. and 24·82 % from the C.E. These figures correspond to 29·47 % and 33·72 % of galactose respectively in the two extracts.

The mother liquors from the mucic acid were examined qualitatively for saccharic acid, but in no case was any found; on the other hand, both tartaric and oxalic acid were shown to be present, the latter in larger quantity in the case of the H.E.

Estimation of pentoses.

Sebor [1900] expressed the view that carrageen mucilage contained a small quantity of pentosan or methyl-pentosan. The H.E. and C.E. were accordingly tested by the Kröber-Tollens method to discover whether pentoses could be discovered in either or both of these fractions. The actual results obtained were: H.E. 1·89 % of pentosan (mean of two estimations); C.E. 1·38 % of pentosan.

Since concentrated solutions of carrageen mucilage will set to a jelly on cooling it was thought that this mucilage might contain pectic bodies. Accordingly a direct hot water extract of *Chondrus* was tested according to the method of von Fellenberg [1914], which consists in steam distilling an alkaline solution of the substance under consideration, and testing the distillate for methyl alcohol and acetone [Tutin, 1921]. Two grams of the extract of carrageen were therefore gently boiled for $6\frac{3}{4}$ hours under a reflux condenser with 100 cc. of $N/10$ NaOH, and the alkaline solution, which had darkened slightly, was then steam distilled. The distillate was practically neutral and slightly yellow in colour. It was tested for alcohol by the acid and bichromate test and for acetone by the iodoform and nitroprusside tests, but the result was negative in each case. This was therefore taken to prove the absence of pectic bodies from the mucilage of carrageen.

Residue left after extraction by cold and hot water.

The residue of the *Chondrus* left after the removal of the C.E. and H.E. showed a great reluctance to part with its last traces of water-soluble material. It was boiled for several hours on a water-bath, the water being changed periodically, but each time this water on being filtered off and tested with Molisch's reagent gave a strong positive reaction. A fresh portion of residue was therefore heated over a Bunsen burner with three successive changes of water, but this treatment also failed to remove the last traces of water-soluble material. Recourse was then had first to a steriliser and finally to an autoclave. After several hours in each of these, however, water-soluble material was still coming out, and was found to contain calcium.

Kylin [1915] claims to have found an insoluble calcium compound in *Chondrus crispus*. He apparently cut sections of fresh weed, boiled them in water and then left them to soak over night, concluding that this treatment removed all water-soluble substances present. He then found calcium to be present in the sections and assumed it to be in an insoluble form. In view of the extreme difficulty experienced during the present investigation in getting rid of the last traces of water-soluble material from the residue of carrageen which had already been soaked for several days in water, it seems unlikely that all was removed by Kylin's treatment. Hence the calcium-containing substance he found was in all probability merely the last traces of the H.E.

Since it was found so difficult to extract the residue completely merely by heating with water, a portion was boiled under a reflux condenser with

3 % NaOH. After two days of this treatment the contents of the flask were filtered, and the residue on the filter-paper washed free from alkali. It was then soaked in dilute hydrochloric acid to remove the carbonate found to have been formed during the alkali decomposition. When washed free from acid the residue was found to be protein- and ash-free, and completely soluble in cuprammonia, showing it to be pure cellulose. Cellulose was also obtained from the residue by acid decomposition. This was effected by boiling for some hours with hydrochloric acid. The solution was filtered and the solid matter left on the filter-paper was washed and tested with cuprammonia. The total amount of cellulose in carrageen, estimated by alkali decomposition, was found to be 1·3 %.

CERAMIUM RUBRUM.

After it had been definitely established that the sulphate in both the C.E. and H.E. of carrageen is present as an ethereal sulphate linked to an organic radicle it seemed probable that this grouping might be found to occur in other seaweeds. Accordingly it was decided to examine another alga which was known to yield an ash with a high sulphate content, and for this purpose *Ceramium rubrum* was selected.

Three grams of dried *Ceramium* were heated with distilled water on the water-bath for an hour. The seaweed was then removed and the liquid filtered and evaporated. Scales of a transparent, horny material were obtained. Some of this material was dissolved in distilled water and the solution was divided into two portions *A* and *B*, barium chloride being added to each. To solution *A* a small quantity of concentrated hydrochloric acid was added while solution *B* was left neutral; both were then boiled for one and a half hours. At the end of this time a precipitate of barium sulphate had been formed in the acid solution *A* only, thus demonstrating the presence of the ethereal sulphate grouping in *Ceramium rubrum*. The investigation is being continued.

General.

Microscopic examination of sections of the thallus of *Chondrus* show that the cell walls become very thick and swollen in distilled water. This is what one would expect if they were largely composed of H.E. and C.E., since on being placed in water dried scales of these materials absorb much moisture and swell up enormously. While only 1·3 % of the dry weight of carrageen consists of cellulose the cell walls appear under the microscope to constitute the greater part of the section, and over 80 % of its weight consists of H.E. and C.E. Since these contain complex polysaccharides they are allied to cellulose and are possibly degradation products of it.

The exact nature of the organic radicle or radicles present in the ethereal sulphates has not been determined. There may be one bi-valent or two uni-valent groups in each molecule (see formula on p. 581).

The present investigation has shown that the two extracts, though differing chemically as well as physically, are nevertheless closely allied. It may be that each extract contains a mixture of various closely related bodies and that different ones predominate in each extract. The similarity of the oxidation products points to both extracts consisting of substances belonging to the same group of compounds, but there is not enough evidence to show whether the differences are due to different bodies or mixtures in different proportions of the same substances. On the whole the former of these alternatives appears the more likely.

Summary.

1. Dialysable organic matter can be separated from the C.E. of carrageen.

2. The C.E. contains calcium and ammonium ethereal sulphates and its ash contains, besides sulphate and calcium, magnesium, sodium, potassium and traces of iron. The ash of H.E. also contains these radicles, but it has less sodium and potassium and more calcium than that of C.E.

3. The ammonium salts of both extracts have been prepared by replacing the ionised calcium by the ammonium radicle.

4. Un-ionised magnesium is present in both extracts.

5. The main oxidation products of both extracts consist of mucic, oxalic and tartaric acids. More mucic acid, but less oxalic acid, is obtained from C.E. than from H.E.

6. Pentose radicles are present in both extracts, but more in cold than in hot.

7. Pectic bodies are absent from both extracts.

8. Cellulose is present in the residue of carrageen left after extraction with both cold and hot water.

9. There are indications that the ethereal sulphate grouping is present in *Ceramium rubrum*.

In conclusion the writer wishes to express her thanks to Dr Haas for his help and advice during the progress of the work, and his kind interest throughout.

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LVIII. STUDIES OF THE COAGULATION OF THE BLOOD.

PART II. THROMBIN AND ANTITHROMBINS.

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In a recent paper in this *Journal* the present writers questioned the current view that antithrombin is secreted by the liver. It was suggested that the action of that organ in promoting the fluidity of the blood after the injection of "peptone" depends on variation of carbon dioxide content of the blood [Pickering and Hewitt, 1921]. Experiments were devised to test this suggestion with the somewhat unexpected result that in pithed cats respiring air, typical inhibition of the coagulation of the blood followed the rapid injection of "peptone" into animals with the liver out of the circulation. Further, the anticoagulant action of "peptone" was annulled by partial asphyxia and was restored by administration of air. It was found also, when suitable precautions were taken to preserve the surface conditions of the blood, that the addition of "peptone" to blood *in vitro* inhibited clotting when the concentration of "peptone" was no greater than was required to produce a like effect *in vivo*.

Immunity to the anticoagulant action of "peptone" was also found to follow the slow injection of that substance into cats with the liver out of circulation [Pickering and Hewitt, 1922].

Having thus demonstrated that the typical action of "peptone" on the blood of the cat can be obtained without invoking the aid of the liver, it became of interest to examine the action of thrombin on the circulating blood of animals under similar conditions, and also to re-examine the evidence for the extraction of antithrombin from the liver, blood and other tissues. Observations on these latter points are presented in this communication.

The Intravascular Injection of Thrombin into animals deprived of Hepatic Circulation.

The thrombin was dissolved in mammalian Ringer's solution and was extremely active as a coagulant *in vitro*; thus the addition of 0·25 cc. of a solution, prepared by dissolving 0·0048 g. of solid thrombin in 100 cc. Ringer,

to 0·75 cc. of cat's blood caused coagulation to commence[1] in 45″ on glass, completion[1] of this process being one minute later. On paraffined surfaces these figures were 45″ and 2′ 30″ respectively.

Experiment 1. Black cat, 2060 g. Pithed. Artificial respiration was maintained for 20′ before the aorta and inferior vena cava were ligatured. The following table shows the coagulation times of blood shed on to glass before and after the injection of thrombin into the circulation.

Table I.

Time	Notes	Commencement of coagulation	Completion of coagulation
0′ 0″	Intact animal	7′ 40″	12′ 0″
1′ 0″	Animal pithed, vessels ligatured	—	—
36′ 30″	— — —	7′ 50″	11′ 48″
36′ 45″	7·5 cc. of 0·0048 % thrombin in Ringer injected	—	—
39′ 0″	— — —	1′ 0″	7′ 10″
44′ 0″	— — —	1′ 10″	7′ 50″
71′ 0″	— — —	2′ 50″	9′ 30″
86′ 0″	— — —	1′ 45″	8′ 50″

Notes: (1) Between 44′ and 86′ several observations were made. The coagulation times given at 71′ show the greatest variations.

(2) *Post-mortem* examination revealed no intravascular clots.

(3) Other experiments of the same nature yielded similar results.

Experiment 2. In this observation 0·01 g. of thrombin was injected into a 2¼ kilo. cat under precisely similar conditions to those of Exp. 1. Similar results were obtained. The subsequent injection of 7·5 cc. of 10 % calcium chloride in distilled water caused no material alteration in the coagulation times of shed blood. A further injection of 5 cc. however killed the animal. *Post-mortem* examination did not show any clots in those portions of the vascular system where blood had been circulating.

These experiments show: (1) That relatively large doses of thrombin can be injected into cats respiring air and with the liver out of circulation without causing intravascular coagulation. (2) That subsequent injection of a lethal amount of calcium chloride in distilled water fails to induce thrombosis. (3) That blood shed after the injection of thrombin into animals deprived of hepatic activity coagulates much more rapidly than the normal blood of the same animal under otherwise similar conditions.

It may be remarked that the present authors have found, in the course of a large number of observations on pithed cats, that if the air supply of the animals is regulated and kept constant, then constant times of commencement and of completion of clotting of shed blood can be maintained for considerable periods.

The great rapidity of clotting of shed blood after the injection of thrombin may in part be explained by the fact that in the experiments recorded above no excretion of injected thrombin by the kidneys was possible; thus, directly the blood was shed, the surface conditions of the plasma were altered and the thrombin was able to exert its coagulant action.

[1] "Commencement" of clotting indicates the first visible departure from normal fluidity: "completion" that coagulation had advanced so far that the vessel could be inverted without spilling.

The somewhat delayed coagulation reported by Davis [1912] following the injection of thrombin into intact animals under ether anaesthesia and preceding the typical hypercoagulability has not been observed in this series of experiments. Unfortunately Davis records only the times of completion of clotting of shed blood after injection of thrombin and does not give the coagulation times before, recording instead the "bleeding times," estimated by the rate of decrease of haemorrhage from a small skin wound, such as in the lobe of the ear. Davis employed the method of Duke [1910]. It should be noted that Weil [1920] has shown that prolonged bleeding time may coexist with normal coagulation time. Moreover Mendenham [1915] found that coagulation is hastened by ether anaesthesia. Unless precautions were taken to maintain a constant depth of anaesthesia and a constant carbon dioxide and oxygen content in the blood slight variations of coagulability, like those observed by Davis, might well occur. These would be masked later by the coagulant action of the thrombin.

The bearing of the foregoing experiments on the "thrombin theories" of coagulation.

The experiments reported in this paper are directly opposed to a commonly accepted view, advocated by Howell [1918], that the injection of thrombin stimulates the liver to secrete an excess of antithrombin, which latter neutralises the injected thrombin and so maintains the fluidity of the blood. They are also dissonant with the suggestions of Howell [1912] "that prothrombin or thrombin itself constitutes a hormone which excites the secretion of antithrombin," and are concordant with the fact that the typical action of "peptone" on blood can be obtained in cats deprived of hepatic activity. Support is thus given to the conclusion [Pickering and Hewitt, 1922] that the retarded coagulability of shed blood after the rapid injection of "peptone" into cats is due neither to the secretion of antithrombin nor of excess of alkali by the liver. This latter view, advocated originally by Dastre and Flouresco [1897], and later by Mellanby [1909] is also dissonant with the recent work of Gratia [1921] who, using Marriott's colorimetric method [Marriott, 1916], was unable to find any evidence of the slightest difference in the alkalinity of normal and "peptone" plasmas.

In the hypotheses of Morawitz [1904, 1] and of Fuld and Spiro [1904], the formation of thrombin from thrombogen (prothrombin) is said to be the prelude of the actual coagulation of the blood. Thrombin, it is postulated, acts directly on the fibrinogen of the plasma and is the immediate cause of clotting.

So long as it was reasonable to assume that the liver secreted sufficient antithrombin to neutralise the effect of massive doses of thrombin these theories remained tenable. As large amounts of thrombin can be injected without thrombosis into the circulation when such hepatic secretion is impossible, the view that thrombin acts directly on the fibrinogen of *unaltered*

plasmas must be discarded and the views of Morawitz and of Fuld and Spiro become untenable. The theory of Howell [1912] explains the fluidity of normal blood by the presence of antithrombin which prevents the calcium of the blood from activating prothrombin to thrombin. The coagulation of shed blood is attributed to the neutralisation of antithrombin by thromboplastin, derived from platelets and tissue cells. Thrombin, so soon as formed, is again regarded as the immediate cause of clotting, a conclusion which is opposed to the evidence brought forward in this communication.

The most recent development of the thrombin theories, that propounded by Bordet [1920], accepts the belief in the existence of antithrombin and other anticoagulants in normal blood, but dissents from the view of Howell as to the rôle played by cytozyme (thromboplastin) in coagulation, citing the work of Gratia [1920] on the neutralisation of hirudin by cytozyme in support of his contention. There is also another important difference between the views of Howell and of Bordet. The former author accords primacy to antithrombin in the maintenance of fluidity, the latter, together with his co-workers [Bordet and Gengou, 1903; Bordet and Delange, 1913], assigns equal importance to surface conditions. Bordet maintains that contact of the blood with foreign bodies causes the interaction of lipoidal cytozyme (thromboplastin), formed largely from platelets but also from leucocytes and other sources, with serozyme, a product of the plasma formed immediately prior to coagulation. Serozyme and cytozyme are then said to combine, in the presence of calcium, to yield thrombin which in turn acts on fibrinogen to give clots of fibrin. Thus apart from the preliminary processes involving the production of thrombin, the first act of coagulation is believed to be the action of thrombin on fibrinogen. The evidence in this paper shows that if the physical conditions of the blood are preserved by remaining in the living vessels, then thrombin, in amounts sufficient to cause coagulation *in vitro*, does not cause thrombosis *in vivo*. It thus appears that although Bordet is correct in assigning importance to the surface conditions of the blood in the maintenance of fluidity, it is not to the disintegration of formed elements yielding thrombin, under the influence of physical change, that the *inception* of coagulation is due, but rather to physical change destroying the stability of the clotting complexes of the plasma. It is only after such change that thrombin can act as a coagulant. In this view, thrombin would be regarded as an accelerator of coagulation rather than as an initiator of that process. The work of Vines [1921] indicates that the complex associated with the inauguration of clotting exhibits distinctive differences from the prothrombin of Howell.

This view of the action of thrombin is supported by the observation of the present writers [Pickering and Hewitt, 1921] that in blood surrounded by oil, coagulation commences as a reversible gel. Further the work of Howell [1916] indicates that the change from a gel to a typical clot is a physical process due to ageing and condensation.

The addition of thrombin to blood in the gel stage immediately transforms the gel into typical fibrin, the speed of coagulation being identical with the speed of clotting when thrombin, in the same concentration, is added to blood shed on to clean glass by means of a paraffined cannula. This latter observation is concordant with the fact discovered by Gratia [1918], that the coagulation of blood induced by thrombin proceeds at the same rate on paraffined as on glass surfaces.

Bürker [1904] found platelets to remain intact in blood on a paraffined surface, yet under these conditions the first stage in coagulation—the formation of a reversible gel—sets in. This falls into line with the observations of Achard and Aynaud [1908] that if coagulation takes place in a moist vaselined chamber at 16–18°, then the platelets remain independent of the fibrin filaments. These authors maintain that physical changes of platelets are the result, rather than the cause of coagulation. Morawitz [1904, 2] found that thrombokinase (cytozyme) could be obtained from platelets. Cramer and Pringle [1913] showed that thrombokinase is not present in oxalate plasma and concluded that recalcification "induces coagulation primarily by causing the breaking up of platelets and only secondarily by influencing the chemical reaction which takes place in the presence of the substance (thrombokinase) liberated by the platelets," also the "filtered oxalate plasma...is readily coagulated by both filtered (through a Berkefeld filter) and unfiltered platelet extract in the presence of soluble calcium salts." On the other hand Gratia [1918] concluded that contact with foreign substances capable of being wetted by blood, does not act solely on platelets and other formed elements but also on the colloids of the plasma. The same writer also states that calcium chloride, if dissolved in distilled water, disintegrates platelets, liberating, presumably, cytozyme (thromboplastin or thrombokinase). Attention is drawn to experiment 2 of this paper in which massive doses of calcium chloride in distilled water were injected into the circulation of an animal deprived of hepatic activity and subsequent to a massive dose of thrombin. In this experiment, if Gratia is correct, platelets were disintegrated; it appears therefore that thrombin plus cytozyme from platelets is unable to cause clotting when the surface conditions of the blood are maintained by contact with the living walls of the blood vessels. It also indicates that platelets in concentrations existing in circulating blood, play only a secondary part in provoking coagulation.

It has been shown that the rapid addition of a tissue extract (a source of cytozyme) to bird's blood *in vitro* has a coagulant action, while the slow addition of similar amounts has an anticoagulant action [Pickering and Hewitt, 1921]. Demonstration has also been given that the intravascular injection of either calcium chloride [Löwit, 1892] or of inactive nucleoprotein [Halliburton and Brodie, 1894], each of which causes leucolysis and therefore liberates cytozyme into the circulation, is not followed by thrombosis.

These facts indicate that the relatively slow liberation of cytozyme by the

disintegration of formed elements in the blood is insufficient to induce thrombosis.

Bloch [1920] suggested that in circulating blood the calcium ions exist in an "inactive and latent state" and that on issuing from the vessels, contact with the *air*, dust and so-called thromboplastic material may transform "inactive" calcium to an active electrolytic precipitant of the colloids of the plasma. The work of Chio [1917] has indicated that the tension of carbon dioxide in the blood may affect the equilibrium of the calcium salts which in turn regulates the physical state of the blood, while Vines [1921] has shown that a change from combined calcium to ionised calcium takes place during clotting.

In Cramer and Pringle's experiments [1913] blood was shed through the air directly into oxalate[1]. If Bloch's view is correct change towards clotting had already taken place. Caution should, it is thought, be exercised in deducing any theory of coagulation from the behaviour of blood after exposure to the air, oxalation and replacement of ionised calcium.

In the experiments of Cramer and Pringle referred to, platelets, leucocytes and some erythrocytes were removed from oxalate plasma, and from these was prepared a "platelet extract" whose coagulant action was tested *in vitro*.

The work of Spring [1900], Höber and Gordon [1904], Paine [1911] and Galecki [1912] shows that the speed of addition of a coagulant electrolyte is an important factor in coagulation of inorganic sols, while the present writers have demonstrated that similar conditions determine the coagulation of bird's blood by tissue extract *in vitro* [Pickering and Hewitt, 1921]. If experiments are to show that lysis of platelets is the initial factor in *normal* coagulation, it would be necessary to prove: (*a*) that the "platelet extract," or the aggregates of platelets, was free from products extracted from leucocytes and erythrocytes; (*b*) that the addition of the "platelet extract" was made at the same rate as the liberation of the coagulant material occurring normally in shed blood; (*c*) that the concentration of either "platelet extract" or of aggregates of platelets added, was not greater than the concentration of extract resulting from the disintegration of platelets in normal shed blood; (*d*) that the lysis of platelets added to oxalate plasma in the presence of ionised calcium took place at the same rate as the disintegration of platelets in normal shed blood.

[1] In a private communication to one of the present authors (J. A. H.) Dr Cramer expressed the opinion that our remarks on p. 719 of Vol. xv of this *Journal* regarding the technique of Cramer and Pringle were liable to misinterpretation; he suggested that we might refer to it in our next communication on the subject in question. We stated "*that blood was not withdrawn through a paraffined or similarly treated cannula.*" The method actually was [Cramer and Pringle, 1913] "*to cut the artery so that the blood flowed directly into the receiving vessel.*" It will be seen that the statement of fact is strictly correct.

The significance of the methods of preparing antithrombins and allied substances.

Doyon and his colleagues Morel, Policard, Dubrulle and Sarvonat [1910–1919] describe the following ways of preparing antithrombin. The liver of the dog is frozen and thawed three times over a period of 48 hours; during the intervals between freezing and thawing it is exposed to the air at room temperature. The liver is then perfused with 0·9 % sodium chloride and dilute alkali carbonate. The perfusate has no anticoagulant action until heated to 100° when it yields antithrombin. Alternative methods involve such processes as autoclaving liver at 120° or exposing minced liver to chloroform vapour or allowing intestine to undergo autolysis.

By the use of one or other of these methods Doyon has prepared antithrombins from the majority of the organs of the body, from the muscles of the cray-fish and from macerated earthworms. Recently however Doyon [1921, 1, 2] finds that antithrombin can only be obtained from the liver of graminivorous birds by freezing and thawing, a process which involves breaking up the colloidal complexes of the cells. In this connection it is noteworthy that Howell ascribes the fluidity of shed bird's blood on glass to excess of antithrombin secreted by the liver. Doyon also found that even freezing and thawing, in the case of the rabbit's liver, failed to produce an anticoagulant; yet Davis [1912] has shown that thrombin can be injected into the circulation of the rabbit, in large doses, without causing thrombosis.

The method adopted by Howell [1914] to demonstrate the presence of antithrombin in the blood was by heating oxalate plasma or peptone plasma to 60°. The filtrate delays the action of thrombin on fibrinogen and is consequently said to contain antithrombin. This antithrombin, Howell states, is destroyed by heating at 80°–85°. Collingwood and Macmahon [1914] found that serum, particularly when kept for two days, exhibits an inhibitory influence on thrombin; this antithrombin is destroyed by heating to 60 or 65°. The retarded coagulability of "peptone" plasma is commonly ascribed [Howell, 1918] to excess of antithrombin secreted by the liver. This antithrombin is completely destroyed only at 100° [Nolf, 1908].

Even allowing a wide margin for differences in stability due to the presence of various protective colloids in the respective solutions and also to individual experimental error, it is difficult to believe that liver antithrombin stable in solution at 100°, or even after autoclaving at 120°, is the same substance as the antithrombin prepared from plasma which is destroyed at 80–85°, or that which appears in disintegrating serum and is destroyed at 60°, or the anticoagulant of "peptone" plasma which is active up to 100°. Yet if the current view is accepted that antithrombin obtained from the liver is actually secreted into the circulation, these anomalies must be ignored.

The most recent method for the preparation of antithrombin is the extraction of rapidly dried and hashed lungs by benzene; the extract is dissolved in

0·9 % sodium chloride and purified by precipitation at its iso-electric point. The anticoagulant substance produced is stated by Mills, Raap and Jackson [1921] to be antithrombin.

The earlier methods of manufacturing antithrombin demand drastic disintegration of the colloidal complexes from which the anticoagulant is prepared. The term "manufacturing" is used as such methods cannot imply unaltered extraction. Moreover, Mills, whose technique is less drastic, admits his method involves removal of a phospholipin from a protein-phospholipin complex, so that again there is only a product of cellular break-down. Likewise in serum, in self-digested viscera and in the exudation of decaying liver exposed to chloroform, the anticoagulant may well be considered as a product of autolysis.

When more physiological methods were employed negative results were obtained. Menten [1920] found that perfusion of the liver with normal saline failed to yield any anticoagulant substance. Minot [1916] applied Doyon's chloroform method to solutions of Howell's antithrombin (prepared from oxalate plasma by heating to 60°), and also to plasma and serum both unheated and heated to 60°. In each case no anticoagulant was found. Loeb [1904] was unable to obtain any antithrombin-like material by extraction of linings of blood vessels, a result which throws doubt on the view, recently revived, that antithrombin is secreted by the endothelium of the vascular wall. Rettger [1909] found no evidence of the production of antithrombin after the serial injection of minimal doses of thrombin into the dog and into the rabbit.

Attempts to cause the liver to secrete antithrombin by means of bile, bile salts, secretin and by electrical stimuli gave negative results [Denny and Minot, 1915]. It is noteworthy that Bulger [1918] found an increased coagulation time in cats, rabbits and guinea-pigs suffering from anaphylactic shock, associated with a decrease of antithrombin estimated by Howell's method. Thus the altered coagulability of anaphylactic shock, which closely resembles that of "peptone" shock, also affords no evidence of the secretion of antithrombin.

In short, no direct evidence has been obtained of the existence of antithrombin in living animals.

Two other organic anticoagulants merit notice. Schikele [1912] stated that the extract obtained from the uterus by pressure retarded coagulation. Bell [1914] repeated these experiments but failed to find any anticoagulant. Howell and Holt [1918] extracted dried powdered liver with ether. The solution thus obtained was precipitated by acetone, redissolved in ether and reprecipitated by absolute alcohol at 50°. This last process was repeated from 12 to 20 times and yielded a substance (or mixture) termed by them *heparin*, which is a powerful anticoagulant *in vivo* and *in vitro*; it differs from antithrombin in that it does not neutralise the action of thrombin. Here again the product obtained appears to be the result of more or less drastic

disintegration of cells. Howell and Holt admit that there is no direct evidence of the existence of heparin in the blood. Nevertheless they believe it is an important factor in the maintenance of the fluidity of the blood, and suggest that it inhibits clotting by preventing the activation of prothrombin to thrombin and also by activating pro-antithrombin with the production of antithrombin.

The preparation of antithrombin from vegetable cells.

In view of the evidence indicating that antithrombins are artificial products formed by the dissolution of complex substances of animal origin, it became of interest to enquire if the employment of Doyon's technique would produce an anticoagulant from vegetable sources.

The raw materials selected were baker's yeast and a very pure crystalline specimen of edestin. It may at once be stated that from both these vegetable products active anticoagulants were prepared.

In each case the materials were suspended in water and heated to 120° under pressure for 4 hours on three separate occasions; when not being heated they were freely exposed to the air. The product was centrifuged and to the clear supernatant liquid sodium carbonate was added until the solution was neutral to litmus. In the case of the yeast the neutralised solution was dried at room temperature at low pressure and was dissolved in water when required.

The action of the products obtained from yeast will be evident from the following experiment.

Blood was shed from a pithed cat respiring air and with the liver out of circulation, through a paraffined cannula into similarly treated vessels. Thrombin was dissolved in 0·9 % sodium chloride. The yeast extract was dissolved in distilled water. 5 cc. of blood were employed in each experiment.

	Blood on paraffined surface	Blood plus 2 cc. distilled water	Blood plus 0·75 cc. 0·5 % thrombin	Blood plus 2 cc. yeast extract	Blood plus 2 cc. yeast extract plus 0·5 cc. thrombin	Blood plus 2 cc. yeast extract plus 0·75 cc. thrombin
Commencement of clotting	26′ 10″	4′ 45″	0′ 45″	Unclotted 2 hours later	24′ 10″	18′ 40″
Completion of clotting	29′ 50″	8′ 45″	2′ 45″	Small clots next morning	43′ 36″	41′ 10″

Note: Similar results were obtained when the yeast extract was dissolved in 0·9 % sodium chloride.

In other experiments of this nature in which non-paraffined vessels have been used, clotting has been observed to occur in fractions. In this, the first clot formed contracted rapidly, while a portion of the fluid remained uncoagulated. Later a second small clot appeared and contracted normally and so on. In clotting induced however by addition of thrombin the clot invariably assumed the typical gel form and later contracted in the normal manner.

Similar results were obtained with rat's blood and with human blood. In the latter case the shed blood had been in contact with cut tissues. With

dog's blood shed through a paraffined cannula into glass vessels, from animals in which the liver was not acting, the anticoagulant action, though evident, was not so pronounced. Employing the same materials as in the experiment just given, delays of 6 and 10½ minutes were obtained in the times of commencement and completion of coagulation.

The action of the yeast extract on the coagulation of recalcified oxalate plasma was investigated.

Fresh oxalate plasma was centrifuged for 30′ at 4000 r.p.m. On recalcification with calcium chloride the times of clotting were: commencement 8′, completion 9′ 15″.

To the same volume of plasma was added 0·192 g. of yeast extract; after solution and recalcification the times were 28′ and 31′ respectively.

Employing the usual animal technique previously described, 0·1 g. of the desiccated material, dissolved in 0·9 % sodium chloride, was injected into the heart. Blood withdrawn 10 minutes after injection yielded a minute clot in 8 minutes, but the great bulk of the blood was fluid after 22 hours. After 26 hours however large gelatinous clots were present. The normal coagulation times of the blood of the animal in question were 8′ 15″ and 9′ 50″ for commencement and completion. Other experiments gave similar results.

The crude material employed in the above experiments was extracted with boiling ether and the ether removed at room temperature. The resultant product was also found to possess well marked anticoagulant properties when tested on recalcified oxalate plasma and on unsalted blood; it did not however neutralise the coagulant action of thrombin added to shed blood. It thus resembles in its action the substance extracted by Howell and Holt from the liver and termed heparin.

Treatment of edestin as mentioned above gave a product which behaved like that obtained from yeast except that the coagulant action of added thrombin was not neutralised.

The preceding experiments show that extract of yeast exhibits the typical characteristics of the antithrombin prepared by Doyon. It not merely acts as an anticoagulant *in vivo* and *in vitro* but antagonises the action of thrombin; this and the preparation of a material resembling in its action heparin, when considered in relation to the methods of obtaining antithrombins from the colloids of cells and of serum, indicate that these substances are not secreted by organisms possessing blood streams but are *post-mortem* products of the break-down of colloidal complexes.

Doyon [1921, 1, 2] has recently shown that the nucleic acids prepared by Neumann's method from thymus and from ganglia are anticoagulants *in vivo* and *in vitro*; he assigns the former action to the secretion of a nucleo-protein by the liver. Experiments to be reported in a subsequent paper have demonstrated that in an animal deprived of hepatic activity the anticoagulant action is equally well marked.

SUMMARY.

1. The massive intravascular injection of thrombin into cats deprived of hepatic activity does not produce thrombosis but accelerates the coagulation of shed blood. A phase of retarded coagulability of shed blood preceding hypercoagulability was not observed when the above conditions were adhered to.

2. The view that the continuance of the fluidity of circulating blood after the injection of thrombin is due to the secretion by the liver of an excess of antithrombin is dissonant with the results of the foregoing experiments.

3. In the theories of Morawitz, of Fuld and Spiro and of Bordet, apart from the preliminary processes involving the formation of thrombin, the first act of coagulation is held to be the action of thrombin on fibrinogen. The evidence in this paper indicates that the inception of coagulation is due to physical change destroying the stability of the colloidal complexes of the plasma. Thrombin appears to be an accelerator of clotting rather than an initiator of that process.

4. The addition of thrombin to blood in the state of a reversible gel causes immediate coagulation.

5. Accepting the statement of Gratia that platelets are destroyed by calcium chloride, it is shown that detritus of platelets, in concentrations not greater than can be formed by lysis in circulating blood, plays only a secondary part in provoking coagulation.

6. Attention is drawn to certain inherent difficulties in interpreting experimental results obtained by the use of "platelet extracts."

7. An analysis of the methods of obtaining antithrombins from liver, sundry tissues and from serum, indicates that the anticoagulant substances so formed are *post-mortem* products.

8. The employment of Doyon's technique for preparing antithrombin from liver, yielded from yeast an extract which exhibited the properties of antithrombin. Further extraction with ether yielded a material similar in its action to heparin. The hydrolysis of edestin also gave an anticoagulant.

9. It is concluded that antithrombins are not phylogenetic products of the animal kingdom arising late in evolution as a protection against thrombosis, but are products resulting from the hydrolysis of protein.

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LIX. INVESTIGATIONS ON THE NITROGENOUS METABOLISM OF THE HIGHER PLANTS.

PART III. THE EFFECT OF LOW-TEMPERATURE DRYING ON THE DISTRIBUTION OF NITROGEN IN THE LEAVES OF THE RUNNER BEAN.

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In any research into the metabolic processes taking place in plants, where fresh material is used, the period during which the research can be carried out must necessarily be restricted to the appropriate season during which the plants can be grown. To overcome the difficulty, thus entailed, some workers have used leaves that had been previously dried, either at low temperatures or from 50–70°.

As far as is known, however—and this statement applies especially to those who have investigated the N and protein metabolism in plants—no one has first investigated the possible changes taking place during drying. Both protein synthesis and degradation are continually going on in the leaf cell; consequently, proteolytic enzymes must be present, and it is strange that so many workers have assumed that these would remain inactive during the process of drying, often at temperatures of 30–50°, the most favourable for enzyme action.

The present research shows that in as far as the leaves of the runner bean are concerned, considerable proteolysis takes place, with consequent increase in the simpler water-soluble nitrogenous products, chiefly ammonia (in the form of ammonium salts or as the amide N of asparagine) and monoamino acids, the former being increased from 1 % to 5 % or 6 % of the total leaf N, the amount of increase depending on the conditions of drying.

On reviewing, therefore, the work done in the past to throw light on the N metabolism in the leaf, it would appear that the predominance given to asparagine as a final product of protein metabolism is based, to a certain extent, on an erroneous conception as to the amount of it actually present in the living leaf.

Boussingault [1860] was one of the first systematically to study the asparagine content of leaves, and he emphasised that it could only be isolated

when the plants were young. His results were confirmed by Meunier [1880] and Müller [1887], who extended their researches over a wide range of plants. Emmerling [1887], who studied the N content of the fresh leaves of *Vicia Faba maj.* throughout the life history of the plant, never found that either ammonia N or asparagine N exceeded 1·25 % of the total leaf N, a result the author of the present paper confirmed in an unpublished series of experiments in 1921. The latter also [Chibnall, 1922] found less than 1 % for each of these substances during the life of *Phaseolus vulgaris* v. *multifloris*. Kosutany [1897] found a similar result for *Vitis vinifera*. Yet side by side with these results others were being published which indicated that the asparagine content of leaves could be quite high. On investigation it would appear that in all these cases the leaves had been dried before analysis. Thus Suzuki [1897] gives 5 % of asparagine in leaves of *Phaseolus vulgaris* dried in an air oven at an unstated temperature. Wassilieff [1901] found 21·46 % of the total N as asparagine in young leaves of *Lupinus alba* dried at 70°. Jodidi, Kellogg and True [1920] found in spinach leaves (age unstated) dried at atmospheric temperature for 7 days, 4·8 % of the total N as ammonia and about 12 % as "amide N of asparagine." Yet the present author found in the fresh leaves from plants three weeks old only 0·6 % as ammonia and 2·05 % as "amide N of asparagine."

From the results given later, combined with those stated above, it seems fairly certain that these high values must be attributed, at any rate in part, to proteolysis during the drying. The same remark must apply to the results obtained by Prianischnikoff [1899], who investigated the seedlings of several legumes, though he analysed the plants as a whole, and not the leaves alone.

Whilst proteolysis thus affects the water-soluble products of the leaf, it is found that the general character of the leaf proteins, as shown by Hausmann's method of analysis, undergoes but little change, if any, during the drying.

Experimental details.

The plant used was *Phaseolus vulgaris* v. *multifloris* (Scarlet Champion). The samples of leaves investigated were from series 8, 9, and 10, of a former research [Chibnall, 1922]. In these the total weight of leaves picked was—in series 8, 2064 g. from 12 plants; in series 9, 2424 g. from 12 plants; in series 10, 1860 g. from 8 plants. For the distribution of N in the fresh leaves only about 500 g. were required, the remainder, in each case, being air-dried in a closed oven through which a slow stream of air was drawn. Series 8 was dried at 50° in approximately 100 hours, series 9 at 40° in approximately 120 hours, and series 10 at 40° in approximately 50 hours. The dried leaves were afterwards ground to a moderately fine powder and stored in a stoppered bottle. About six months elapsed before the present research was commenced.

Table I gives further details of the air-dried samples. As the drying took place in the dark, the leaves of series 8, which had been picked one hour before dawn, received no opportunity for photosynthetic action before death.

Table I. *Showing details of the air-dried samples used.*

Series number	Approximate age of plant above ground in weeks	Time of day when picked (G. M. T.)	Fresh weight of sample in g.	Air-dried weight of sample in g.	% of moisture in air-dried sample
8	11	2 a.m.	1365	213·0	7·65
9	11	3 p.m.	1925	335·1	10·92
10	14	9 a.m.	921	161·8	7·17

General methods of manipulation (*separation of protein, soluble products and cellular matter*). The method of grinding in an end-runner mill, as used with the fresh leaves [Chibnall, 1922], was found to be applicable in the case of the dried leaves. The ground up particles of cellular matter imbibe the water and swell up, so that only the very fine ones pass through the pores of the lawn when gentle pressure is applied. By using lawn of very close texture, as in the case of series 8, this can be reduced to a minimum, the percentage of N per dry-weight of the colloidal protein being but very little lower than that from the fresh leaves. Even when moderately coarse lawn is used, so as to allow the separation to be conducted in the minimum of time, the amount of carbohydrate mixed with the protein is not sufficient to interfere with the chemical analysis of the latter (*vide* series 9 in Table IV).

In some preliminary experiments the ground leaves were first shaken up with a small quantity of water and allowed to stand for some hours, with the object of facilitating the extraction of the less soluble products, but subsequent analysis of the extracts, as will be shown later, indicates the presence of proteolytic enzymes, which are activated by the addition of water. It is therefore necessary to carry out the extraction in the minimum of time.

In the preparations from 50 g. of the dried leaves of series 8 and 9, upon which the subsequent analyses given herein are based, 6 extractions by grinding, followed by six extractions with boiling water were given. Extraction with boiling water without the preliminary grinding in the cold was also tried. The protein- and proteose-free water-soluble N agreed with that given by the other method, but only a part of the proteoses passed into solution and, of course, no colloidal protein was obtained. On account of lack of material, the analysis given for series 10 in Table IV was of an extract prepared by this method. Table II illustrates the difference between the extracts prepared by the two methods.

Table II. *Showing the water-soluble N extracted from the dried leaves by cold and hot extraction.*

(In percentages of total leaf N.)

Series number	Number of extractions		Total water-sol. N	Precipitated by ammonium sulphate		Protein- and proteose-free water-soluble N
	By cold grinding	With hot water		Not resoluble in water (protein?)	Proteose N	
8	6	6	30·78	0·51	3·62	26·65
8	0	10	27·65	0·48	1·45	25·71
9	6	6	33·68	0·39	3·74	29·55
9	0	10	30·94	0·28	0·69	29·97

Analytical methods. The details given in the former paper were followed, with the following exceptions:

Proteose N. This was estimated by ammonium sulphate instead of zinc sulphate in acid solution [Baumann and Bömer, 1898]. No difficulty had been experienced with the latter method when using the extracts from the fresh leaves, but in those from the dried leaves the zinc sulphate separates a quantity of non-nitrogenous material of a slimy nature, which renders the subsequent filtering slow, and the washing of the separated proteoses almost impossible. No difficulty was experienced with the ammonium sulphate, the proteoses filtering readily at the pump. They were then washed with a saturated solution of ammonium sulphate until the washings were colourless and redissolved in water. The operation was repeated, and the N of the aqueous solution of the proteoses, after expulsion of the free ammonia by distillation with magnesia *in vacuo*, was determined by Kjeldahl's method. The N of the small insoluble residue on the filter paper, probably coagulated protein, was also determined, after thorough washing, by Kjeldahl's method.

Residue after extraction of the colloidal protein and water-soluble products. As this still contained about half the protein N of the leaf, three extractions with 700 cc. of 1 % HCl were given, the extracts being united and analysed as before.

Results.

Effect of drying on the proteins. Table III gives a detailed account of the proteins extracted from the dried leaves of series 9, with the corresponding data for the fresh leaves. The amount of protein passing into colloidal solution is proportionately much smaller than with the fresh leaves, but could no doubt be increased by further grindings, since the 6th extract appeared as heavily charged with colloidal matter as the earlier ones. The bulk of the protein not passing into colloidal solution, however, is extracted by the HCl. Assuming, as in the case of the fresh leaves, that the small amount of N in the final residue of cellular matter is due to unextracted protein, it will be seen that the total protein N has fallen from 74·83 % to 61·15 % of the total leaf N.

Table III. *Comparison of the proteins and water-soluble N in the fresh and dried leaves of series* 9.

(In percentages of total leaf N.)

	Fresh leaves	Dried leaves
Colloidal protein extracted	59·70	29·44
Protein extracted by 1 % HCl	10·24	25·00
Remaining in residue	4·13	5·90
Coagulated by boiling	1·76	0·42
Coagulated by saturation with ammonium sulphate (?)	0·00	0·39
Total protein N	74·83	61·15
Proteose N	4·35	3·74
Protein- and proteose-free water-soluble N	17·22	29·55
Extracted by alcohol and ether	3·60	2·96

Table IV gives the distribution of N in the colloidal proteins from the fresh and dried leaves of series 8 and 9. It will be observed that no apparent change has taken place in the protein molecule.

Table IV. *Comparison of the distribution of N in the colloidal proteins from the fresh and dried leaves.*

(In percentages of total protein N.)

Series number	% of N per dry weight	Amide N	Humin N	Basic N	Monoamino N	Other N
8 fresh	11·75	6·14	3·55	19·33	59·25	11·73
8 dry	10·11	6·26	4·63	21·20	57·93	9·98
9 fresh	11·71	6·34	3·64	22·00	58·88	9·14
9 dry	8·64	6·73	4·71	21·67	56·30	10·51

Table V gives the distribution of N in the proteins extracted by the HCl from the fresh and dried leaves of series 9. In comparing them it must be remembered that they represent very different proportions of the total leaf protein. The HCl extract from the fresh residues represents only about $\frac{1}{6}$ of the total protein, that from the dried leaves nearly $\frac{1}{2}$. Since the leaf cell undoubtedly contains more than one protein, it follows that if the dried leaves, like the fresh, contain a small amount of protein rich in amide and basic N, these characteristics, on analysis, may be masked by the predominance of unextracted protein, of the type represented by the colloidal protein. If allowance be made for soluble carbohydrates due to the mild hydrolytic action of the HCl on the pentosans etc. of the cell wall material, which will increase the humin N at the expense of the monoamino N, the distribution of N in the HCl extract from the dried leaves does not differ from that of the corresponding colloidal protein, indicating that the major part of it is of this type. Interpreting the various distributions of N in terms of the amounts of the total leaf protein that they represent, it would appear that the leaf proteins do not undergo any appreciable change in character during the process of drying.

Table V. *Comparison of the distribution of N in the proteins extracted by* 1 % *HCl.*

(In percentages of total N in the extract.)

Series number	Amide N	Humin N	Basic N	Monoamino N	Other N
9 fresh	8·19	5·82	24·66	48·78	12·64
9 dry	6·96	6·16	21·57	54·01	11·30

Effect of drying on the proteoses. Table VI shows that in the cold extracts prepared from the dried leaves the proteoses have diminished. Since Table II shows that they are but slowly soluble in hot water, it may well be that after drying they are not so readily soluble in cold water, in which case the apparent loss may be due to incomplete extraction.

Table VI. *Comparison of the distribution of N in the aqueous extracts from the fresh and dried leaves.*

(In percentages of total leaf N.)

Series number	Total water-soluble N	Protein- and proteose-free water-soluble N	Ammonia N	Amide N of Asparagine	Nitric N	Humin N	Basic N	Mono-amino N	Other N
8 fresh	21·07	*	0·36	0·0	3·56	1·00	*	7·06	2·34
8 dry	30·78	26·65	6·02	0·0	3·57	1·15	1·81	10·55	3·55
Diff.	9·71	—	5·66	—	—	—	—	3·49	1·21
9 fresh	21·57	17·22	0·44	0·53	2·73	0·99	2·00	7·76	2·77
9 dry	33·68	29·55	5·32	1·51	1·62	0·82	2·84	13·22	4·22
Diff.	12·11	12·33	4·88	0·98	1·11	—	0·84	5·46	1·45
10 fresh	22·75	18·16	0·37	0·47	3·53	1·06	2·30	6·81	3·62
10 dry	(1)	27·07	2·68	2·26	3·01	0·89	3·51	10·60	4·12
Diff.	—	8·91	2·31	1·79	0·52	—	1·21	3·79	0·50

* Proteoses not determined.

(1) Determined from an extract made with hot water only, which gives a low value for the proteoses.

Effect of drying on the protein- and proteose-free water-soluble products. Table VI shows that in all three cases this has increased, the amount being roughly proportional to the time of drying. The products of the protein autolysis fall chiefly into ammonia (as ammonium salts or "amide N of asparagine") and monoamino acids. This is illustrated better by Table VII. Leaving for a moment the relation between the ammonium salts and asparagine, which is discussed later under the heading of enzymes, it will be seen that in the two samples picked after some hours' daylight, series 9 and 10, the amount of ammonia N is nearly equal to the monoamino N, whereas in series 8, picked after 6 hours' dark, the ammonia is in considerable excess. In the latter case this may possibly be due to the higher temperature of drying (50° instead of 40°) or, more probably, to the fact that the leaves, before drying, were deprived of most of their reserve carbohydrate by translocation away from the leaf at night.

Table VII. *Showing the ammonia and amino acids formed by proteolysis during drying.*

(In percentages of total autolysed protein N.)

Series number	Ammonia N + amide N of asparagine	Monoamino N	Total: {Ammonia N / Amide N of asparagine / Monoamino N}
8	58·3	35·9	94·2
9	47·5	44·3	91·8
10	46·0	42·5	88·5

In series 9 and 10 again, there is a loss of nitric N, probably due to the necessary carbohydrate for the synthesis of organic nitrogenous products being present, since in series 8 there is no loss. The higher temperature

of drying may have accounted for this, however, since Couperot [1909] has shown that leaves dried at room temperature lost 25–50 % of their nitric N as hydrocyanic acid whereas those dried rapidly at 60° suffered no such loss.

Table VIII. *Showing the enzyme action produced by allowing the dried leaves to stand with cold water.*

(In percentages of total leaf N.)

Series No.	Hours standing with water	Total water-soluble N	Protein and proteose N removed by ammonium sulphate	Protein- and protease-free water-soluble N	Ammonia N	Amide N of asparagine	Nitric N	Humin N	Basic N	Monoamino N	Other N
8	0	30·78	4·13	26·65	6·02	0·0	3·57	1·15	1·81	10·55	3·55
8	16	35·90	3·16	31·74	2·30	2·96	3·59	0·89	3·76	13·56	5·68
9	0	33·68	4·13	29·55	5·32	1·51	1·62	0·82	2·84	13·22	4·22
9	16	39·35	3·39	35·94	1·69	4·58	1·67	0·90	5·63	15·80	5·67
9	116	39·82	2·86	36·96	1·20	4·10	1·56	1·04	5·67	17·48	5·91
10	0	*	*	27·07	2·68	2·26	3·01	0·89	3·51	10·60	4·12
10	16	31·60	3·21	28·38	1·19	3·57	3·04	1·02	3·33	11·14	5·09

* Determined in a hot water extract only, which gives a low value for the proteoses.

Enzymes present in the dried leaves. As was stated earlier, if the ground leaves are allowed to remain suspended in water for some hours (under a layer of toluene), proteolytic enzymes become active, with consequent increase of the simpler nitrogenous products in solution. Table VIII shows the analyses that have been made to illustrate this. A study of the figures shows that the action is complex, but the following points stand out fairly clearly:

(1) There is at first a rapid increase in the water-soluble N tending to reach a limit with lapse of time.

(2) Monoamino N shows a continuous increase.

(3) Basic N shows an increase that is not maintained with lapse of time.

(4) Ammonia N shows at first a rapid decrease, tending to reach a limit. At the same time there is a nearly corresponding increase in the "amide N of asparagine."

(5) The sum ammonia N + "amide N of asparagine" shows a slow decrease, indicating re-synthesis of more complex bodies.

From (2) and (3) it is clear that an enzyme of the nature of a pepsin is present (the extract is slightly acid). But the chief point of interest is the rapid conversion of the ammonia N into the amide N of asparagine. In the leaf the origin and relationship between these two substances have not yet been definitely established. Under normal conditions the evidence is that the concentration of both of them is low. Whether the ammonia is due only to direct translocation from the root, or to protein degradation in the leaf itself or to both of these is not yet clear. Undoubtedly an enzyme capable of breaking down the protein into ammonia and amino acids exists in the dried leaves, but this may not be so in the normal fresh leaf. In a previous paper [Chibnall, 1922] there was described an experiment in which the living leaf

was starved under conditions that precluded the translocation of the products of metabolism, and in spite of considerable protein degradation, no appreciable increase in either ammonia or "amide N of asparagine" was noted[1]. Suzuki [1897], by starving moist leaves for 48 hours, claims to have increased the "asparagine N" from 5 % to 13 % of the total N, but his results must be queried since he dried the leaves before analysis.

Asparagine itself cannot be a primary product of protein degradation, but must be a product of re-synthesis from the ammonia and amino acids. In very young plants direct translocation of it from the cotyledon reserve is possible, since Prianischnikoff [1896] showed in germinating seeds of *Vicia sativa* a steady increase of asparagine at the expense of the protein. Butkewitsch [1909], who experimented with seedlings of lupins and peas, considers, as does Suzuki [1894], that the formation of asparagine is to remove an injurious excess of free ammonia. This view, if modified to include an excess of monoamino-dicarboxylic acids also, is very likely, and the reaction "ammonia + aspartic acid $\rightleftarrows$ asparagine" is probably due to an enzyme governed by the H ion concentration of the medium in which it is working. As far as is known, an enzyme of this nature, an asparaginase, has not yet been indicated, and it is therefore of great interest to observe that one appears to be present in the dried leaves used in this research. Furthermore, under the conditions of the present experiment, it appears to have marked synthetic activity.

It is hoped at an appropriate season to repeat these enzyme experiments with fresh leaves, and if an asparaginase should be indicated, to study the effect of change in the H ion concentration upon its action.

In conclusion the author would like to thank Prof. S. B. Schryver for his advice and encouragement throughout the research, which was made possible by a grant from the Department of Scientific and Industrial Research.

Summary.

1. The effect of low-temperature drying on the nitrogenous bodies in the leaves of the runner bean has been investigated.

2. Protein autolysis takes place, with increase in the simpler water-soluble N products.

3. These products are chiefly ammonium salts, asparagine and amino acids.

4. The leaf proteins, whilst they are diminished in amount, are not appreciably changed in character.

5. Proteolytic enzymes are present in the dried leaves, and are activated by the addition of water.

[1] The season 1921 was abnormal, the bean plants bearing but few pods. At the time this experiment was carried out, 23 August 1921, no pods were in process of formation. Further starvation experiments are being carried out this season, and the results so far to hand from leaves picked in August 1922, when pod formation was active, indicate that considerable quantities of asparagine are formed by degradation of the leaf protein. Ammonia N, as before, is unchanged.

6. The presence of an asparaginase, activated by the addition of water, is indicated. Under the conditions of the present research it possesses marked synthetic activity.

7. The position of ammonia and asparagine in the N metabolism of the leaf is discussed.

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LX. INVESTIGATIONS ON THE NITROGENOUS METABOLISM OF THE HIGHER PLANTS.

PART IV. DISTRIBUTION OF NITROGEN IN THE DEAD LEAVES OF THE RUNNER BEAN.

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(*Received July 12th, 1922.*)

In a former paper [Chibnall, 1922, 1], the seasonal variations in the N content of the leaves of the runner bean, *Phaseolus vulgaris* v. *multifloris* (Scarlet Champion), were demonstrated and discussed. The period examined covered the first twenty weeks of the plant's life, from the seedling stage, until the leaves were showing signs of chlorophyll degeneration. Eleven samples were analysed, numbered consecutively "series 1–11."

The plants died during the 24th week, following two nights' hard frost, and the present communication deals with the distribution of N found in the dead leaves, which will be denoted henceforth as series 12. As picked from the dead plants they were shrivelled up and dry, whilst their green colour was masked by a brownish-red stain, probably due to iron salts, since it was easily washed away with cold water.

The results given later for series 12 have in all cases been compared with the corresponding ones for series 9, 10 and 11, the four together covering the latter half of the plant's life, from the 11th to the 24th weeks. During this period the plants were fully grown and pod formation was complete, so that the general metabolism would be, presumably, on the down grade. The tables given later show how remarkably stable is the general equilibrium between the nitrogenous substances in the leaf during this period, the changes, even at death, being of quite a small order. There is no evidence of any great withdrawal of N from the leaves before death, nor is there any accumulation of asparagine. Miyachi [1896] assumed that asparagine accumulates in the aged leaves of *Paeonia albiflora* since old leaves from dying plants, if placed under water for 14 days, become rich in this substance. In the present author's opinion this experiment merely demonstrates the presence of active enzymes in the old leaves. (Compare Chibnall [1922, 2], where observations on dried leaves suspended in water are recorded.)

The methods of manipulation and analysis were those used for dried leaves, as described by Chibnall [1922, 2].

The results can be summarised under the following headings: (1) There is no great withdrawal of N from the leaves to the stems or roots when the plant becomes aged [*vide* Czapek, 1920]. (2) The protein- and proteose-free water-soluble N of series 12 shows a slight increase, due chiefly to ammonia (as ammonium salts or "amide N of asparagine") and monoamino N. From the author's experiments on the changes in the leaf N due to low-temperature drying [Chibnall, 1922, 2], it would appear that this increase has taken place during the period of dehydration of the leaf on the plant. Towards the latter end of the plant's life the nitric N has fallen, as one would expect from the ageing of the root system, otherwise the equilibrium between the water-soluble nitrogenous products has remained very steady, indicating a period of low metabolic activity in the leaf. (3) The proteins in the leaf have undergone slight amidisation, a change that must be ascribed to the ageing of the leaf, since it was not observed in the proteins of leaves dried at low temperatures. The ratio of protein to non-protein N in the leaf has suffered no appreciable change with age or death.

Table I. *Showing the percentage dry weight and total N in the leaves of series* 9–12.

Series number	Age above ground in weeks	% dry weight	% of N per fresh weight	% of N per dry weight
9	11	15·51	0·675	4·35
10	14	16·31	0·724	4·44
11	20	16·13	0·622	3·86
12	24	91·6	—	3·60

Table II. *Showing the amounts of protein and non-protein N in the dead leaves* (*series* 12).

(In percentages of total leaf N.)

Colloidal protein extracted	29·22
Protein extracted by 1 % HCl	29·49
N in residue (protein?)	6·62
*Protein coagulated by ammonium sulphate ...	0·77
Total protein N	66·10
Proteose	6·28
Proteose and protein-free water-soluble N ...	22·10
Soluble in alcohol and ether	2·15
Total N accounted for	96·63

* In estimating proteoses by saturation with ammonium sulphate, a small amount of N, probably due to traces of coagulated protein, is not resoluble in water.

Table III. *Showing the water-soluble N in series* 9–12.

(In percentages of total leaf N.)

Series number	Total water-soluble N	Proteose N	Coagulated protein N*	Protein- and proteose-free N
9	21·57	4·35	—	17·22
10	22·75	4·57	—	18·16
11	22·41	4·38	—	18·03
12	29·15	6·28	0·77	22·10

* As in footnote for Table II.

Table IV. *Showing the distribution of the water-soluble N in series* 9–12.

(In percentages of total leaf N.)

Series number	Total protein- and proteose-free N	Ammonia N	Amide N of asparagine	Nitric N	Humin N	Basic N	Mono-amino N	Other N
9	17·22	0·44	0·53	2·73	0·99	2·00	7·76	2·77
10	18·16	0·37	0·47	3·53	1·06	2·40	6·81	3·62
11	18·03	0·61	0·92	1·28	0·96	2·59	6·61	5·06
12	22·10	0·97	1·94	1·61	1·32	2·22	7·74	6·30

Table V. *Percentage distribution of N in the colloidal proteins of series* 9–12.

Series number	Amide N	Humin N	Basic N	Monoamino N	Other N
9	6·34	3·64	22·00	58·88	9·14
10	6·50	3·82	21·78	59·22	8·68
11	6·40	3·82	21·32	58·50	9·96
12	7·21	6·12	19·27	56·22	11·32
12*	8·81	6·67	19·27	51·90	13·35

* HCl extract of series 12.

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LXI. NOTE ON THE NON-PROTEIN NITROGEN IN GOAT'S MILK.

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(*Received June 28th, 1922.*)

As a result of an investigation into the factors controlling the percentage composition of milk, during which a goat was fed on various diets each of which was abnormally high in some one of the three energy-yielding constituents of the food, it was found that irrespective of the nature of the diet the percentages of protein, fat, lactose and ash all tended to vary with the daily volume of milk secreted, the percentages of protein, fat and ash tending to vary inversely and the percentage of lactose directly with the daily volume [Taylor and Husband, 1922]. So far as these constituents are concerned, therefore, the percentage composition of milk depends upon its rate of secretion rather than upon the percentage composition of the diet.

In the case of non-protein nitrogen no relationship could be traced between the percentage and the daily volume of the milk. The results of the following experiment show that the percentage of this constituent depends upon the nature of the diet.

Experimental.

A goat was fed on various diets of constant composition for periods varying from 8 to 16 days, and the total protein, caseinogen and albumin plus globulin contained in the milk were estimated daily. The figure for non-protein nitrogen was obtained from the difference between the percentage of total protein and the sum of the percentages of caseinogen, albumin and globulin. The figure for non-protein nitrogen is expressed in terms of milk protein, as the non-protein nitrogen was included in the estimation of total nitrogen from which the total protein was calculated.

The urine was collected and a daily estimation of the total excretion of urinary nitrogen was also carried out.

The following table shows, for each dietary period, the average daily total quantity of nitrogen in the urine and the average percentage of non-protein nitrogenous substances in the milk. The periods are arranged in the table according to the level of nitrogen excreted in the urine.

The table shows a definite correlation between the daily output of nitrogen in the urine and the percentage of non-protein nitrogen in the milk.

Average daily excretion of urinary nitrogen g.	Average percentage of non-protein nitrogenous substances in the milk per cent.	Nature of diet	Duration of experimental period Days
26·8	0·39	High protein	16
22·5	0·37	„ „	12
9·6	0·22	Potatoes	8
9·4	0·22	Grass	14
4·3	0·20	High fat	14
4·1	0·20	„ „	14
2·3	0·16	High carbohydrate	13

Discussion of Results.

It has been shown that the non-protein nitrogenous substances in milk consist of amino-acids, creatine, creatinine, uric acid, and urea, the last named being present in greatest amount [Denis, Fritz Talbot and Minot, 1919]. The concentration of these in the blood seems to determine both the amount excreted in the urine and the percentage present in the milk. The mammary gland acts to some extent as an excretory organ, waste non-protein nitrogenous substances filtering through from the blood to the milk. The percentage in which these are found in milk seems to be determined by the degree of concentration in the blood of the end products of protein metabolism.

Conclusions.

1. In a lactating animal there is a correlation between the daily output of nitrogen in the urine and the percentage of non-protein nitrogen in the milk, both apparently being determined by the amount of protein in the food.

2. The mammary gland acts as an excretory organ passing through from the blood to the milk end products of protein metabolism.

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LXII. SMELL.

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(*Received July 3rd, 1922.*)

THE aim of this investigation was to find a method of accurately and scientifically describing smells. Sounds and colours can be accurately described by the frequencies of the vibrations of which they are composed, but no such method has yet been discovered for describing smells, and the best that can be done at present is to say that there is some kind of resemblance between the smells of certain substances though at the same time it is generally felt that such a description is very unsatisfactory and incorrect. Not only is it impossible to describe any smell satisfactorily but there is at present no criterion as to whether a smell is simple or complex. It is true we can sometimes detect more than one substance in a mixture by the smell but we are generally helped in such an analysis by the different volatility of the different constituents of the mixture, and in fact such analysis is very uncertain and liable to error.

The problem is difficult, because, so far as is known at present, smell is not due to any kind of vibration such as the air vibrations causing sound or the ether vibrations causing light. The fact that most and probably all odorous substances are volatile and that no odorous substance can be detected by its smell when enclosed in an air-tight receptacle made of any ordinary material has led to the general belief that contact between the odorous substance and the olfactory organ is necessary to cause the sensation of smell. At any rate temporarily the author has assumed that smell is due to chemical or physical reaction between the odorous substance and the olfactory organ. It is well known that certain odorous substances react chemically with proteins, *e.g.* the halogens, nitrous fumes, hydrochloric acid, formaldehyde. And as the olfactory organ is undoubtedly composed chiefly of proteins it seemed worth while first to see whether any chemical reaction could be detected between proteins and odorous substances in general. The method used by Bugarsky and Liebermann [1898] to prove chemical reaction between non-coagulated white of egg and hydrochloric acid seemed specially suitable. These investigators showed that the addition of egg-white to aqueous hydrochloric acid caused an elevation of the freezing-point, pointing to a decrease of the number of hydrochloric acid molecules in the solution. A similar experiment was therefore tried by the present investigator with aqueous ethyl acetate solution and white of egg but no elevation of the freezing point was observed. An experiment was also tried by shaking solid egg-albumin (Merck's) with aqueous ethyl acetate to see whether the amount of ethyl acetate was reduced. But the egg-albumin, although not readily

soluble in water, was sufficiently soluble to make filtration very difficult. The experiment was therefore modified by using alcoholic solutions of various odorous substances, shaking them up with egg-albumin and ascertaining whether the amount of odorous substance in solution had decreased. At any rate it was expected that acids and bases would react with the egg-albumin. A decrease was observed in the case of hydrochloric acid but none in the case of ethyl acetate, acetic acid, pyridine or citronellal. It is obvious, therefore, that under these circumstances there is no chemical reaction between several typical odorous substances and the typical protein, egg-albumin.

In looking round for a physical reaction between odorous substances and the olfactory organ which might be the cause of smell the author was struck by the parallel between intensity of smell and depression of the surface tension of the aqueous solutions of various organic substances investigated by Traube. Physiologists give much attention to the problem of how chemical substances can enter the living cell, for it is obvious that no reaction can take place with the cell-substance unless entry can be obtained into the cell. Surface tension and adsorption are likely to play an important part in bringing substances into contact with the living cell, and it was therefore very interesting to notice in Traube's results that the strongest smelling substances produced the greatest depression of the surface tension in aqueous solutions.

But before much progress could be made in detecting a parallel between smell and depression of surface tension it was necessary to find a method of measuring the intensity of smell, and this seemed likely to be a very difficult matter as common experience seems to indicate that different animals have very different sensibilities as regards smells. Dogs by their sense of smell can obviously detect things which are quite inodorous to human beings, and one human being seems to have a much keener sense of smell than another. Moreover it seemed likely that the intensity of smell of a substance would depend among other things on its vapour pressure. Practical psychologists describe an instrument in which air is inhaled by the nostrils over a definite area of filter-paper surface moistened by a solution of the substance to be tested. But before experimenting with this instrument the simpler plan was tried of making aqueous solutions of different strengths of the substance to be tested and ascertaining the minimum strength of solution in which the substance could be detected by smelling the solution contained in an ordinary bottle at the ordinary temperature. Almost contrary to expectation it was found that consistent results could be obtained in this way, and that several persons obtained practically the same results. Details are given in the experimental part.

These determinations of the intensity of the odour of the substances examined by Traube confirmed the conclusion previously suspected, viz. that those substances had the strongest smell which produced the *greatest depression* of the surface tension in aqueous solution. The intensity of odour and also the surface tension of aqueous solutions of additional substances were deter-

mined, especially of essential oils and other substances well known for their strong odours, and with a few exceptions the above rule was found to hold. The constituents of the well-known perfumes, *e.g.* citral and geraniol, produced a very great depression of the surface tension. Out of 22 substances examined it was found that 17 would be arranged in exactly the same order whether they were arranged according to *increasing depression* of surface tension or increasing intensity of odour. The list is as follows: formic acid, methyl alcohol, acetic acid, ethyl alcohol, propionic acid, methyl acetate, methylamine, phenol, ethyl acetate, butyric acid, *iso*-amyl alcohol, quinoline, cinnamic aldehyde, citral, allyl sulphide, geraniol. The most noteworthy exceptions were ammonia, pyridine and ethyl mercaptan which did not depress the surface tension to the extent anticipated from their powerful odours.

The method used for the determination of surface tension was of the simplest character and it is well known that such determinations are liable to large experimental errors. Rather than spend more time on eliminating possible errors in surface tension determinations it was decided to make some determinations of adsorption. According to theory adsorption should run parallel with surface tension and it is obvious that from the point of view of contact of the odorous substance with the olfactory organ we are more interested in adsorption than in depression of surface tension which was merely used as an index of adsorption.

Arranged according to adsorption from aqueous solution by animal charcoal two of the exceptions above noted disappear, viz. pyridine and ethyl mercaptan, which have adsorptions in keeping with the intensity of their odours.

Arranged according to depression of surface tension	Arranged according to adsorption by charcoal	Arranged according to intensity of odour
Ammonia	Ammonia	Formic acid
Formic acid	Formic acid	Acetic acid
Acetic acid	Acetic acid	Propionic acid
Ethyl mercaptan	Propionic acid	Ammonia
Pyridine	Pyridine	Butyric acid
Propionic acid	Butyric acid	Pyridine
Butyric acid	Ethyl mercaptan	Ethyl mercaptan
	Citral	Citral

We may therefore enunciate the general rule that those substances have the strongest smell which are most readily adsorbed from aqueous solution.

It was thought that by replacing animal charcoal by a protein we might obtain a closer approach to the olfactory organ. Some experiments on adsorption by wool from aqueous solutions were tried but did not lead to any result of interest.

Although, apparently, no progress has been made in solving the problem outlined at the beginning of this paper, it is interesting to have found a connection between smell and physical properties capable of exact measurement, and it does not seem too much to hope that it may prove a first step in solving the problem which the author has set before him.

Experimental.

Determination of the minimum concentration of aqueous solution in which odorous substances can be detected by smell.

The solutions were all placed in similar bottles which were stoppered and shaken just before they were smelt by bringing the nose close to the mouth of the bottle. In order to avoid fatigue the more dilute solutions were presented to the observer before the more concentrated ones and in order to avoid the influence of suggestion he was not told what solutions were being presented to him. The results obtained by the author, two Indian colleagues (Dr A. C. Sircar and Babu S. C. Ganguli) and one Indian student (Babu J. K. Mazumdar) are recorded. It was more difficult to obtain consistent results with the fatty acids than with the other substances examined.

The substances are arranged in order of increasing intensity of odour.

Substance	Percentage strength in which detected by			
	E. R. W.	S. C. G.	J. K. M.	A. C. S.
Formic acid ...	0·1	1	1	—
Acetic acid ...	0·1 (0·3)	1 (0·1)	0·1	—
Propionic acid ...	1 (0·1)	1 (0·03)	0·3	—
Aniline	0·1	—	—	—
Methyl alcohol ...	0·1 (0·01?)	0·1	0·01	—
Ethyl alcohol ...	0·1	0·1	0·1	—
Methyl acetate ...	0·1	0·03	0·1	—
Chloroform	—	0·1	—	—
Butyric acid ...	0·01	0·1 (0·003)	0·1 (?)	—
Ammonia	0·01	—	—	0·01
Methylamine ...	0·01	—	—	—
Ethyl acetate ...	0·01	0·01	0·01	—
Phenol	—	0·01	—	—
Pyridine	—	<0·001	—	—
Quinoline	0·001	—	—	—
Isoamyl alcohol ...	0·001	0·001	0·001	—
Cinnamic aldehyde ...	0·001	—	—	—
Nitrobenzene ...	0·001	0·001	—	—
Ethyl mercaptan ...	0·0001	—	—	—
Citral	0·0001	0·0001	—	0·0001
Geraniol	—	0·0001	—	—
Allyl sulphide ...	0·0001	—	—	—
Phenyl isocyanide ...	0·00001	—	—	—

Determination of surface tension of aqueous solutions of odorous substances.

This was done by observing the rise of the solution in a capillary tube.

In the following list the substances examined are arranged according to effect on surface tension, those having least effect being placed first:

1. Ammonia
2. Formic acid
3. Methyl alcohol
4. Acetic acid
5 and 6. Ethyl mercaptan / Ethyl alcohol
7. Pyridine
8. Propionic acid
9. Methyl acetate
10, 11, and 12. Methylamine / Phenol / Aniline
13. Nitrobenzene
14. Ethyl acetate
15. Butyric acid
16. Phenyl isocyanide
17. Isoamyl alcohol
18. Quinoline
19, 20, and 21. Allyl sulphide / Citral / Cinnamic aldehyde
22. Geraniol

Adsorption from aqueous solution by animal charcoal.

The determination was easy for acids and bases, which can readily be estimated by titration. An approximately normal solution (50 cc.) was shaken with 2 g. animal charcoal (Merck's, washed and dried) and allowed to stand with occasional shaking over two nights, then filtered and titrated. Similarly 100 cc. of an approximately decinormal solution were shaken with 1 g. animal charcoal.

Substance	Titration value (in cc. N soln.) of 10 cc. Original solution	Final solution
Formic acid	21·7 × 0·939	19·9 × 0·939
	17·7 × 0·1108	16·1 × 0·1108
Acetic acid	16·8 × 0·939	14·9 × 0·939
	13·3 × 0·1108	11·55 × 0·1108
Propionic acid	12·3 × 0·939	10·25 × 0·939
	10·3 × 0·1108	8·15 × 0·1108
Butyric acid	8·7 × 0·939	6·6 × 0·939
	6·65 × 0·1108	4·35 × 0·1108
Ammonia	12·4 × 1·036	11·4 × 1·036
	12·3 × 0·11036	11·35 × 0·11036
Pyridine	9·7 × 1·036	7·6 × 1·036
	10·3 × 0·11036	7·3 × 0·11036

Citral. It was found that citral in very dilute aqueous solution such as required for these experiments could be estimated by extracting with ether and titrating the ethereal extract with Hübl's solution.

A solution of 0·3555 g. citral in 1 litre of water was shaken with 10 g. animal charcoal and allowed to stand for 24 hours. The smell of citral had entirely disappeared, so that concentration had been reduced to less than 0·000001.

A solution of 0·4 cc. citral dissolved in 1 litre was shaken with 2 g. charcoal and allowed to stand for 24 hours. The smell had almost entirely disappeared.

An estimation gave the approximate results:

Concentration in water = 0·00000256
„ charcoal = 0·1782

For the same concentration of butyric acid in charcoal we can calculate:

Concentration in water = 0·00201
„ charcoal = 0·1782

So that the adsorption of the citral is very much greater.

Ethyl mercaptan. An attempt was made to estimate ethyl mercaptan in aqueous solution by precipitating with lead acetate, filtering off, washing and weighing the lead mercaptide. It was not very satisfactory owing to the solubility of lead mercaptide.

Ethyl mercaptan (2 cc.) was dissolved in 250 cc. water.

(*a*) 40 cc. of this solution precipitated with 1 g. lead acetate dissolved in 15 cc. water; precipitate filtered off and washed with a measured quantity of water, 10 cc. at a time = 0·2800 g. (corresponds to 0·1055 mercaptan).

(*b*) 10 cc. of mercaptan solution diluted to 40 cc. and treated with 1 g. lead acetate solution, etc., exactly as above, using same quantity of wash water; lead mercaptide obtained = 0·0472 g.

(*c*) 50 cc. of mercaptan solution was shaken with 0·1 g. animal charcoal filtered and 40 cc. of filtrate treated exactly as above; lead mercaptide obtained = 0·0463 g.

(*d*) 50 cc. of mercaptan solution was shaken with 0·5 g. animal charcoal, filtered and 40 cc. filtrate treated exactly as above; lead mercaptide obtained = 0·0122 g.

Although the method of estimating mercaptan is unsatisfactory we can safely say that 40 cc. of the filtrate from (*c*) contained about the same quantity of mercaptan as 10 cc. of the original solution, so that

Concentration in water = 0·00166
,, charcoal = 2·502

From these data we can arrange the substances according to their adsorption by animal charcoal, those which are most adsorbed being placed last in the list: ammonia, formic acid, acetic acid, propionic acid, pyridine and butyric acid (bracketed together), ethyl mercaptan and citral (bracketed together).

The work described in the paper was carried out in the second half of 1919.

REFERENCE.

Bugarsky and Liebermann (1898). *Pflüger's Arch.* **72**, 51.

LXIII. STUDIES ON THE PITUITARY. I.

THE MELANOPHORE STIMULANT IN POSTERIOR LOBE EXTRACTS.

By LANCELOT THOMAS HOGBEN and FRANK ROBERT WINTON.

(*Received July 4th, 1922.*)

Introduction.

To the manifold physiological responses evoked by extracts of the posterior lobe of the pituitary gland, it has lately become necessary to add that of inducing pigmental changes in those lower vertebrates (Fishes, Reptiles, Amphibia) which possess considerable capacity for temporary modification of bodily colour through the activity of a special type of effector organ, the chromatophore (melanophores, etc.). Recent work on the part played by endocrine organs in amphibian metamorphosis has in particular brought to light a number of interesting data relative to the factors which control pigment response in these organisms. One of the most characteristic consequences of pituitary removal in tadpoles noted by Allen [1917] and others was a condition of extreme pallor resulting from general contraction of the dermal melanophores (black pigment cells). More recently Swingle [1921] has shown that implantation of the pituitary in pale tadpoles induces a darkening of the skin, a result shown to follow injection of posterior lobe extracts into adult frogs by the writers [1922]. The present communication attempts to explore the use of frog melanophores as indicators of pituitary extracts and to emphasise the remarkable specificity of this reaction. Few people are aware how marked is the capacity for pigmental change possessed by the common frog (*R. temporaria*), the same individual varying from a light yellow to a coal black or dark grey tint within comparatively short time limits: the difference is mainly due to the "expansion" and "contraction" of the dermal melanophores. In general between temperatures of 0–25° frogs placed in bright illumination are pale if kept dry whether on a light or dark background. They may become dark if placed on a dark background in water. For the study of pigmental darkening by pituitary injection, it is best to leave the animals singly in dry glass dipping jars for a few hours in bright light on a white background to ensure maximal contraction of the melanophores. Observations can be supplemented by microscopic preparations of skin, fixed in Bouin's fluid, dehydrated, cleared and mounted without more elaborate treatment. In earlier experiments [Hogben and Winton, 1922] decerebrated

animals were used. For injection Burroughs Wellcome's all glass tuberculin syringe graduated in 0·1 cc. was found to be most convenient: unless otherwise stated the intraperitoneal method was adopted.

In these experiments the frogs were stored in white porcelain tanks with glass tops, and were thus always ready for use. To facilitate observation individuals in which the skin is uniformly of a yellowish hue when pale, rather than the varieties with irregular markings, spotted or speckled aspect should be selected.

Quantitative Experiments.

The following experiments were carried out to determine how far the melanophore response might be utilised to estimate the activity of posterior lobe extracts. The preparations employed were Burroughs Wellcome's 0·5 cc. phials of liquid sterile posterior lobe (21 %) extract ("infundin") stated to be standardised by action on the isolated mammalian uterus. Injection with 0·5 cc. of 1/1000 solution of this preparation invariably invoked the characteristic darkening of the skin, while 0·5 cc. of a 1/10,000 solution failed to do so in every case. The following preliminary experiments in which the animals employed were not weighed may be quoted to indicate the range of sensitivity of the frog's melanophores to pituitary extracts and the relative sensitivity with different methods of injection. The average temperature was 72° F.

Sample A. Three series of six frogs received an intraperitoneal injection equivalent to 0·00031, 0·00025, 0·00019, 0·00012, 0·00009 and 0·00006 cc. of infundin respectively. In two series the animals which received 0·00012 cc. and upwards underwent darkening of the skin and in the other series those which received 0·00019 cc. and upwards gave a positive reaction.

Sample B. Two series of six frogs were injected with 0·00025, 0·0002, 0·00015, 0·0001, 0·000075, 0·00005 cc. of infundin. In one series (intraperitoneal) the individuals which received 0·00015 cc. and upwards showed visible darkening of the skin accompanied, as seen on microscopic examination, with full expansion of the melanophores. The individual which received 0·0001 cc. displayed the melanophores in a slightly expanded (stellate) condition, while in the remaining frogs the melanophores were fully contracted. In the remaining series (dorsal lymph sac injection) microscopic examination revealed a precisely similar seriation, but the frog which received 0·0001 cc. showed a slight visible darkening which was not noticeable in the corresponding animal of the preceding group.

Sample C. Six frogs received an intraperitoneal and six an intravenous injection equivalent respectively to 0·0005, 0·0004, 0·0003, 0·00025, 0·0001 and 0·00005 cc. of B.W. liquid sterile extract. In both series the animals which received 0·0005, 0·0004 and 0·0003 cc. doses reacted with full expansion of the melanophores; a slight darkening of the frogs injected with 0·00025 and 0·0001 cc. was visible, but microscopic examination revealed the expansion of the melanophores in the former though not in the latter case. The animals injected with a dose of 0·00005 cc. did not react at all.

In these experiments summarised in Table I the frogs used were in no way selected, and it may be inferred that such methods are suitable for detecting the presence of posterior lobe extracts in quantities exceeding that equivalent to 5×10^{-5} g. of fresh gland (ox) substance, *i.e.* about 0·05 % of a clinical dose. In view of the relation between sensitivity and size indicated below it will be seen that by selecting sufficiently small individuals (or species of Amphibia), it should be possible to detect posterior lobe secretions in considerably smaller quantities than this and therefore in amounts that lie much beyond the limits of sensitivity of methods at present employed. We have found no evidence of a difference in the sensitivity of the reaction with the three methods of injection described.

Table I.

Sample	Method of injection	Minimal dose cc.	Subminimal dose cc.
A	Intraperitoneal	0·00012	0·00009
A	Intraperitoneal	0·00012	0·00009
A	Intraperitoneal	0·00019	0·00012
B	Intraperitoneal	0·00015	0·0001
B	Dorsal lymph sac	0·00015	0·0001
C	Intraperitoneal	0·00025	0·0001
C	Intravenous	0·00025	0·0001

Sample D. Two series, each of six frogs, were weighed and arranged in a heavy and a light series. Doses were injected so that pairs, one from each series, received the same dose per unit body weight—the results are shown in Table II, and demonstrate proportionality between body weight and the minimal effective dose.

Table II.

Approximate dose per 20 g. weight cc.	Heavy series			Light series		
	Wt. g.	Dose cc.	Response	Wt. g.	Dose cc.	Response
0·00025	31	0·00037	Darkening	16	0·0002	Darkening
0·0002	30	0·0003	,,	15	0·00015	,,
0·00015	25	0·0002	,,	15	0·00011	,,
0·00015	23	0·00018	,,	14	0·0001	,,
0·0001	23	0·00012	—	13	0·00007	—
0·00005	21	0·00005	—	12	0·00003	—

Sample E. (See Table III.) Eight frogs of 18–20 g. body weight received 0·0002, 0·00016, 0·00014, 0·00012, 0·0001, 0·00008, 0·00006, 0·00004 cc. infundin (intraperitoneal). The first five showed darkening: the rest remained pale, *i.e.* the minimal dose to produce maximal expansion of the melanophores lay between 0·0001 and 0·00008 cc. Six other frogs weighing 15–16 g. were injected *via* the peritoneum with 0·0002, 0·00015, 0·0001, 0·000075, 0·00005, 0·000025 cc. infundin. The first four only showed darkening of the skin, *i.e.* the minimal dose lay between 0·000075 and 0·00005 cc. A third set of eight frogs weighing 13–14 g. were tested from the same sample of infundin with the following doses (intraperitoneal): 0·0002, 0·00015, 0·0001, 0·00008, 0·00006, 0·00004,

0·00002, 0·00001 cc. The melanophores were fully expanded in the first three and contracted in the last three, while the frogs which received 0·00008 and 0·00006 cc. were found to display the pigment cells in a condition of partial expansion.

Sample F. (See Table III.) The following doses were administered to twelve individuals. A 1 (30 g.) and A 2 (25 g.), 0·00035 cc.; B 1 (30 g.) and B 2 (26 g.), 0·0003 cc.; C 1 (30 g.) and C 2 (26 g.), 0·00025 cc.; D 1 (29 g.) and D 2 (27 g.), 0·0002 cc.; E 1 (29 g.) and E 2 (28 g.), 0·00015 cc.; and F 1 (28 g.), and F 2 (28 g.), 0·0001 cc. infundin. The pairs A, B, C, D, uniformly displayed an intense darkening of the skin after half an hour had elapsed. The two pairs E and F on the other hand remained pale. This set of animals was deliberately chosen for large size; none of the females were carrying ripe eggs at the time.

The results obtained from the three foregoing experiments, although obtained with three different samples of "infundin," and therefore heterogeneous, present sufficient uniformity to be instructive in connection with the problem of what order of error is introduced in the method. It will be seen that the interval between minimal and submininal doses, representing the margin of uncertainty, can usually be reduced to 25 %–30 % of the minimum effective dose; occasionally however, as in the third series of experiment (*E*), unsuitability of frogs may extend this even to 60 %.

That the simple correlation between body weight and minimal dose does not invariably obtain, is illustrated by a further experiment which was undertaken with a view to determining the sensitivity of the reaction to a dose injected about two hours subsequent to a previous dose in the neighbourhood of the minimal dose.

Sample G. (See Table III.) A preliminary experiment with four frogs showed that this sample was somewhat more potent than some used previously, and suggested the doses described. 32 frogs were weighed, and divided into four groups.

Eight frogs (9–11 g. body weight) were injected respectively with 0·0002, 0·00015, 0·000125, 0·0001, 0·000075, 0·00005, 0·000025, 0·00001 cc. infundin. Visible darkening of the skin occurred in the first five, the remaining three were unaffected. The minimal dose was in this case between 0·000075 and 0·00005 cc.

Eight frogs (12–13 g. body weight) were injected with 0·00025, 0·0002, 0·00015, 0·000125, 0·0001, 0·000075, 0·00005, 0·000025 cc. infundin. The first three only showed visible darkening of the skin. The minimal dose was thus between 0·00015 and 0·000125 cc.

Eight frogs (14–15 g. body weight) were injected with the same series of doses. The first four in this instance displayed visible pigmental change, the dose necessary being between 0·000125 and 0·0001 cc.

Eight frogs (17–19 g. body weight) were injected with 0·00035, 0·0003, 0·00025, 0·0002, 0·00015, 0·0001, 0·000075 and 0·00005 cc. The first three

only showed darkening of the skin, *i.e.* the minimum dose for this series was between 0·00025 and 0·0002.

Table III.

Sample	Wt.	Actual dose: Min.	Actual dose: Submin.	Wt.	Dose per 20 g. body weight: Mean min.	Dose per 20 g. body weight: Min.	Dose per 20 g. body weight: Submin.	Error in min.
	g.	cc.	cc.	g.	cc.	cc.	cc.	%
D	23	0·00017	0·00012	23	0·00015	0·00015	0·0001	0
D	14	0·0001	0·00007	13				
E	18	0·0001	0·00008	20	0·00012	0·0001	0·00008	− 17
E	15	0·000075	0·00005	16		0·0001	0·00007	− 17
E	13	0·0001	0·00004	14		0·00015	0·00006	+ 25
F	29	0·0002	0·00015	29	0·000145	0·00014	0·0001	− 3
F	27	0·0002	0·00015	28		0·00015	0·00011	+ 3
G	19	0·00025	0·0002	17	0·000215	0·00025	0·00023	+ 14
G	15	0·000125	0·0001	14		0·00017	0·00015	− 21
G	12	0·00015	0·000125	12		0·00025	0·00021	+ 15
G	8	0·000075	0·00005	10		0·00019	0·0001	− 11
G (repeat)	8	0·000075	0·00005	10		0·00019	0·0001	− 11
G (repeat)	17	0·0002	0·00015	18		0·00023	0·00017	+ 7

Mean deviation 11·8 %.

Effect of subsequent doses. After injection with concentrated extracts the condition of melanophore expansion may last 12 hours or more. After injection with minimal doses it passes off in about $1\frac{3}{4}$–$2\frac{1}{2}$ hours. After $1\frac{3}{4}$ hours following the first injection, the last series injected with sample *G* received corresponding doses of the same magnitude. The minimal dose was slightly lower, 0·0002–0·00015 cc. On injecting the first series two hours after the initial treatment the minimal dose was the same as before. It thus appears: first that given sufficient time for recovery the sensitiveness of the melanophores is not appreciably changed by previous treatment, second, that the effect of repeated doses is, in contrast with the action of the pressor principle [Howell, 1898], identical.

Like Dale and Laidlaw's original method[1] for estimating the uterine stimulant, this method is appropriate to comparison rather than absolute standardisation of extracts. Several factors which may lead to anomalous results might be considered, such as possible seasonal variation of sensitivity[2], and variations depending on the immediate previous history of the frogs with respect to moisture, temperature, etc. Again, different strains of frogs may respond with varying facility, and we have certainly noted that some are more and others less satisfactory in giving a sharply defined end point and well contrasted reaction. Furthermore, isolated frog's skin placed in Ringer shows the characteristic response to posterior lobe extracts, and may react with more constant sensitivity than an intact animal; these and other relevant considerations we have at present no opportunity to examine. The specificity,

[1] In Dale and Laidlaw's [1912] standard method for estimating the activity of pituitary extracts by their action on the isolated guinea pig's uterus, the minimal dose of infundin which produces a maximal contraction varies between ·003–·005 cc. which is diluted to about 100 cc.

[2] Compare the striking variation in the electrical phenomena of frog's skin which occurs during the breeding season—[Bayliss and Bradford, 1886].

certainty and delicacy of the melanophore response will recommend it for such biochemical and pharmacological purposes as do not require a degree of accuracy higher than that we have described, namely consistency of minimal dose, to the extent of a mean deviation of 12 %. In each of the foregoing experiments the actual number of frogs used was the minimal number requisite for demonstrating the end point in a series. It is evident that in testing samples on a large scale the degree of activity might be compared with greater confidence by employing a larger number of individuals. In any case a simple and direct method is provided for testing the retention of activity of pituitary products employed in the dispensary or hospital.

Specificity of the Melanophore Stimulant.

Concerning the mode of action of the melanophore stimulant there is no need to add anything further in this place to our previous observations except to emphasise the local response obtained on isolated strips of skin placed in Ringer's extracts. It is our intention to publish in the near future a comprehensive account of the reactions of frog melanophores to drugs; but it is of special interest to insert at this point a few observations under this heading to lay stress on the highly specific nature of the response evoked in amphibian melanophores by posterior lobe extracts. The physiological effects hitherto described as characteristic of posterior lobe extracts are equally characteristic of a group of other well-known drugs. In the case of the melanophore reaction described in this communication we have to deal with a property of the extract which is not precisely simulated by any of the more familiar pharmacological reagents, as the accompanying table signifies. The case of digitalis merits special comment. The water-soluble digitalin (Parke Davis) was employed. In a dosage of 1 mg. death followed 30–45 minutes after injection *via* the dorsal lymph sac and no colour change resulted. 0·5 mg. was generally fatal after a few hours and accompanied in one case by a partial expansion of the melanophores. Doses of 0·3 mg. (*i.e.* one half the minimum lethal dose)

Table IV.

Pale frogs were injected with doses of the drugs undermentioned.

(*a*)	Pilocarpine	25 mg., 12·5 mg., 2·5 mg.	Remained pale
(*b*)	Atropine	10 mg., 3 mg., 1 mg.	,,
(*c*)	Strychnine	1 mg., 1/25 mg., 1/100 mg.	,,
(*d*)	Curare (decerebrated)	Curarised	,,
(*e*)	Histamine	3·6 mg., ·36 mg., ·036 mg.	,,
(*f*)	Ergatoxin	6·5 mg., ·65 mg., ·065 mg.	,,
(*g*)	Tyramine	20 mg., 2 mg., ·02 mg.	,,
(*h*)	Veratrine	1/4 mg., 1/20 mg., 1/100 mg.	,,
(*i*)	Digitalis	Numerous doses of varying range below M.L.D. (cf. text)	,,
(*j*)	Barium chloride ...	1 mg., 1/5 mg.	,,
(*k*)	Sodium nitrite ...	5 mg.	,,
(*l*)	Caffeine	10 mg., 2 mg.	,,
(*m*)	Apocodeine	2·5 mg.—5 mg. without motor paralysis	,,
		10 mg. motor paralysis within 30 mins.	Slight darkening
(*n*)	Nicotine	1/10 mg. no motor paralysis	Remained pale
		1/5 mg.—1 mg. with motor paralysis ...	Partial darkening

or less produced absolutely no effect on coloration. But no effect was obtained on the isolated skin. A partial expansion usually but not invariably occurs with nicotine and apocodeine only in dosage sufficient to produce motor paralysis without immediate lethal effects. In none of these cases is the reaction readily comparable with the pituitary reaction.

Some Chemical Properties of the Melanophore Stimulant.

Experiments were carried out to test how far the chemical properties of the pituitary melanophore stimulant agree with those of the other active constituents of the posterior lobe extracts.

Inactivation by boiling with dilute HCl. There appear to be at least two autacoid substances contained in extracts of the posterior lobe, since it is possible to destroy or reduce the pressor activity of "infundibular" extracts without diminishing in a corresponding manner their power to excite plain muscle. Dudley [1919] by extraction of dried and powdered infundibulum with acidified water, treatment of the solution with colloidal ferric hydroxide, and subsequent continuous extraction of the filtrate at reduced pressure with butyl alcohol, succeeded in separating a residue containing all the uterine stimulant ("oxytocic" principle) together with a portion of the pressor substance. The latter has been shown by Abel and Nagayama [1920] and Dale and Dudley [1921] to be rapidly destroyed by boiling with 0·5 % HCl. The oxytocic principle on the other hand is only slowly destroyed by such treatment. In this respect it appears that the melanophore stimulant is like the uterine principle and is not identical with the pressor substance.

The effect of continued boiling with dilute HCl was investigated as follows. A 0·5 % solution of the commercial extract was made up in 0·5 % HCl. This mixture was subjected to continuous boiling for five hours, a sample being removed at the end of thirty minutes. At the conclusion of the experiment a sample of the unboiled mixture, the portion which had been subjected to only half an hour's hydrolysis, and the residue were respectively neutralised with soda and diluted to a concentration approximately isotonic with frog's Ringer. From each of the three solutions, A (unboiled), B (boiled half an hour), C (boiled five hours), 0·5 cc. was injected into a pair of frogs whose pigment cells were fully contracted. The macroscopic and microscopic examination of the six animals at the conclusion of an hour revealed a marked contrast. The A and B pairs were dark and showed the typical pituitary reaction. The pair C remained pale. Microscopic preparations of the skin showed that in the C pair the melanophores were fully contracted, and the A and B pairs expanded. The result of the experiment indicates that pituitary extracts retain a considerable potency to induce melanophore response after half an hour's boiling with 0·5 % HCl; hence the melanophore stimulant is not identical with the pressor substance. According to Abel and Nagayama the oxytocic activity of the pituitary extracts after being reduced to about one-fifth by hydrolysis

for 30 minutes resists further destruction. Dale and Dudley however did not find that the residue was stable to prolonged boiling with acid; and the behaviour of the melanophore stimulant in this experiment conforms to their experience of the uterine stimulant.

The oxytocic potency of the samples taken after 30 minutes' boiling with 0·5 % HCl was found by Dale and Dudley to be between a quarter and a fifth of the previous activity. The potency of pituitary extract to induce melanophore expansion after half an hour's hydrolysis appears to be reduced in somewhat similar proportions. In a further experiment an approximation to the amount of melanophore stimulant remaining after half an hour's boiling with 0·5 % HCl was investigated thus. Three portions *A*, *B*, *C* (15 cc.) were taken from a 2 % "infundin" solution. *A* was boiled for half an hour, after which 15 cc. 1 % HCl and sufficient 1 % soda to neutralise it were added. To *B* were added 15 cc. of 1 % HCl, which was neutralised before boiling for 30 minutes. *C* was acidified with 15 cc. of 1 % HCl, boiled for half an hour, and subsequently neutralised. Each solution thus contained originally 0·3 cc. of sterile extract. *A* and *B* were made up to two litres so that an injection of 0·3 cc. would be equivalent to 0·00015 cc. infundin. *C* was made up to half a litre: one-half of this (*C* 1), representing a dilution one quarter of *A* and *B*, was used for injection, the other half was diluted one in four (*C* 2). Twenty-eight frogs were injected as in the following table from which it appears, on making the correction necessary for body weight, that the samples *A* and *B* (controls) were about *four* times as potent as *C*, the solution which had been subjected to boiling with 0·5 % HCl.

The positive sign signifies darkening of the skin, the numbers in brackets refer to the body weight in grams, and the extreme left hand column gives the dosage in cc. injected.

Table V.

	A	*B*	*C* 1	*C* 2
0·8 cc.	+ (12)	+ (12)	+ (12)	+ (11)
0·7 „	+ (13)	+ (13)	+ (13)	− (11)
0·6 „	+ (14)	+ (13)	+ (14)	− (11)
0·5 „	+ (16)	+ (16)	+ (16)	− (11)
0·4 „	+ (17)	+ (18)	+ (17)	
0·3 „	− (18)	+ (19)	− (18)	
0·2 „	− (20)	− (20)	+ (20)	
0·1 „	− (23)	− (23)	− (23)	

Action of proteoclastic enzymes. Perhaps the most significant fact indicative of the type of chemical compounds to which the infundibular autacoids are allied is furnished by the action of proteoclastic ferments. Schafer and Herring [1906] stated that pepsin destroys the pressor activity of pituitary extracts leaving intact the diuretic principle. They denied further that trypsin affected either. Later work by Dale [1909] and Dudley [1919] indicates that trypsin rapidly destroys both the pressor and oxytocic principles. To test the action of proteoclastic ferments on the melanophore stimulant six solutions were made up: *A* (0·5 % infundibular extract in 0·2 % HCl and

0·5 % pepsin); *B* (0·5 % infundin in 0·2 % HCl); *C* (0·2 % HCl and 0·5 % pepsin); *D* (0·5 % infundin in 0·5 % saline trypsin); *E* (0·5 % saline trypsin); *F* (0·5 % infundin in 0·5 % *boiled* trypsin). After two hours' digestion *A*, *B* and *C* were neutralised, boiled and diluted till isotonic with Ringer. *D*, *F* were boiled. On injection into pairs of pale frogs, the pairs injected with *A*, *B* and *F* showed darkening of the skin with expansion of the melanophores. The rest remained pale with the melanophores contracted. Thus the melanophore stimulant is rapidly destroyed by trypsin and is not rapidly destroyed by pepsin. In this connection evidence is provided that the melanophore stimulant is not identical, as Abel and Kubota [1919] once believed the uterine principle to be, with histamine, since the latter (Dudley) is not destroyed by tryptic digestion. As a matter of fact histamine does not in any concentration cause melanophores to expand.

Relation to other pituitary autacoids. In view of the possible identity of the diuretic and pressor substances, and the likelihood that the oxytocic stimulant is responsible for the galactogogue action of posterior lobe extracts, it is not necessary to postulate the existence of more than two distinct autacoids secreted by the juxtaneural epithelium, It is possible that the melanophore stimulant is not identical with either the pressor or the oxytocic autacoid, but there is strong evidence that it is not identical with the former, namely (*a*) its slow rate of inactivation by boiling with dilute hydrochloric acid; (*b*) no reversal of response after successive action; (*c*) failure of drugs which agree with pituitary extracts in their effect on the blood pressure to evoke the same reaction. For the present therefore we may provisionally attribute the melanophore response to the oxytocic substance.

Source of the Pituitary Melanophore Stimulant.

In Swingle's [1921] experiments upon the implantation of the pituitary gland in amphibian larvae, the pars intermedia was found to be responsible for the darkening of the skin. The results obtained from injections are consonant with the conclusion that the juxtaneural epithelium is the source of the secretion which induces melanophore expansion.

The demarcation between the three parts of the gland in the case of ox pituitaries is very striking; and ox pituitaries secured within an hour of killing were therefore employed in the following experiment. The three portions of the gland were carefully separated: each portion was weighed, ground up with sand, and extracted with Ringer's solution at 35° for two hours. A 0·1 % and a 0·02 % solution were made up from each extract so prepared. A pair of frogs were injected with each of the six solutions. At the end of half an hour the two pairs injected respectively with the strong and weak anterior lobe extracts remained pale. The pair injected with a weak extract (0·02 %) of pars nervosa extract also remained pale. While the pairs injected with both weak and strong extracts of pars intermedia as well as the pair injected with the stronger pars nervosa extract were conspicuously

darkened through, as microscopic examination displayed, the general expansion of the dermal melanophores. In view of the non-glandular character of the pars nervosa, and the higher concentration of the melanophore stimulant in the pars intermedia, it seems likely that the melanophore stimulant is secreted by the latter portion of the gland, diffusing rapidly into the former.

Phyletic Distribution of the Melanophore Stimulant.

The following observations indicate the occurrence of the same melanophore stimulant in the pituitary gland of various classes of Vertebrata.

Mammalia. An adult female rabbit was decapitated, and its pituitary gland together with pieces of muscle, brain, ovary, pancreas, liver, suprarenals and spleen removed instantly. The tissues mentioned were severally ground with sand and extracted with Ringer in the usual way, after having been first weighed. A pair of frogs was injected with 0·5 cc. per individual of a 1 % solution of each extract. An additional pair received a corresponding amount of a putrid meat extract, while another pair received 0·5 cc. of a 0·1 % solution of the same pituitary extract. The two pairs injected with 1 % and 0·1 % pituitary extracts alone displayed a visible darkening of the skin, and, upon microscopic examination, the expansion of the melanophores.

Birds. Pituitaries were removed from eight ducks, weighed, ground up with sand, extracted and made up to 2 %, 0·4 %, and 0·08 % solutions which were administered respectively to pairs of pale frogs (0·5 cc. per individual). The animals injected with the two stronger solutions displayed the characteristic pigmentary response: the pair injected with the 0·08 % extract however remained pale.

Reptiles. Into each of a pair of frogs an injection of an extract of the pituitary of the lizard (*Lacerta viridis*) was made. The characteristic reaction was evoked. Two other frogs injected with an extract of brain tissue as controls, remained pale.

Amphibia. The experiment with frog's pituitary is of special interest as bearing on the rôle of the juxtaneural epithelium in normal pigmentary responses. Six frogs' pituitaries were removed and made up to 5 cc. in Ringer's solution. An injection of 0·5 cc. into each of a pair of pale frogs evoked an intense darkening of the skin. The remaining solution was diluted 1·5 in 10; and a pair of frogs injected with 0·5 cc. of this solution also underwent darkening of the skin. The 1·5/10 solution was again diluted 1/5. A pair of frogs injected each with 0·5 cc. of the latter showed in one case a darkening, while the other individual remained pale. On further dilution no response was induced by injection. It appears from the foregoing data that the amount of pituitary melanophore stimulant in the gland of one frog is not only sufficient to account for the darkening of the skin in one animal of the same species but in as many as fifty-six individuals.

Fishes. Pituitaries were removed from six cods (*Gadus*) and were made up to 2 %, 0·2 % and 0·04 % extracts. Two pairs of frogs injected with the usual amount of the two stronger solutions displayed the characteristic

darkening of the skin. Only one of two individuals injected with 0·5 cc. of the 0·04 % extract underwent pigmentary darkening: a pair injected with a 5 % extract of cod's brain remained pale.

Tunicata. The widespread distribution of the pituitary melanophore stimulant in the Vertebrate series encouraged the search for a similar substance among the lower Chordata; and extracts of the so-called dorsal tubercle or subneural gland of the Tunicate, regarded by morphologists as the homologue of the Vertebrate hypophysis, were accordingly prepared with this end in view. *Ascidiella*, a solitary Ascidian about 1½ inches in length, was selected for the purpose. The dorsal tubercle in this form is very large and quite conspicuous through the translucent mantle after the gelatinous test has been removed. The organ in question was removed from twenty-four individuals and a 10 cc. extract was made. Injection into pale frogs was not followed by any pigmentary change.

Reaction of Chromatophores to Pituitary Extract.

The observations above mentioned refer to the behaviour of the dermal melanophores of the frog, and in their reactions to pituitary extracts these resemble those of other Amphibia, of which we have examined Hyla, Bombinator (Anura) and Amblystoma (Urodela). The frog possesses in addition at least three other types of pigmentary effector organs: (*a*) epidermal melanophores, responding to pituitary extracts in a manner similar to the dermal melanophores, though their sensitivity may be different; (*b*) the dermal xantholeucophores (yellow pigment cells) which respond *in the opposite sense, i.e.* contracting after pituitary injection—this property not being shared by Hyla in which the xantholeucophores appear to be non-contractile; (*c*) the retinal pigment cells. Fujita and Bigney [1918] have shown that these expand after treatment with adrenaline, an observation which we have confirmed. Pituitary injection has no effect upon them either in the expanded or contracted condition.

The fact that the frog itself possesses two types of chromatophores which differ diametrically in their mode of response to posterior lobe extracts is of interest in view of the fact that Spaeth [1917] describes the contraction of the melanophores of the fish (*Fundulus*) after action of the pituitary autacoid in certain circumstances. In view of the conformity of data derived from glandular extirpation and injection in the case of Amphibia, Spaeth's observations require in our belief reconsideration, though the above-mentioned fact does not permit one to regard his conclusions as necessarily improbable.

Summary.

1. The pars intermedia of the mammalian pituitary secretes a specific stimulant inducing expansion of the dermal melanophores in Amphibia: this property is shared by extracts from the pituitary of birds, reptiles, amphibia and fishes: it was not found in the subneural (hypophysial) gland of Tunicates. Sufficient can be extracted from the gland of one frog to induce intense visible darkening in more than fifty other pale individuals of the same species.

2. The action of pituitary extracts on melanophores is one of extreme delicacy, and can be used to detect with certainty the presence of posterior lobe secretions in quantities equivalent to 5×10^{-5} g. fresh glandular substance (*i.e.* about 1/2000th ordinary clinical dose) by their power to induce intense darkening of the skin in pale frogs.

3. A method of approximate quantitative estimation of potency of posterior lobe extracts is indicated by experiments on the sensitivity of the melanophore response of frogs, which further show within what limits of error such a procedure may be successful. The delicacy of response does not depend on the mode of injection, nor is it affected by previous injection of minimal doses of the extract; it is however correlated with body weight.

4. Whereas the physiological effects hitherto described as characteristic of posterior lobe extracts are equally characteristic of a group of other well-known drugs (*e.g.* digitalis, $BaCl_2$, histamine), the melanophore response described above is a property which is not precisely simulated by any of the more familiar pharmacological reagents. An effect in the same sense is indeed produced only by nicotine and apocodeine in doses sufficient rapidly to produce motor paralysis, and even here the darkening is much less conspicuous.

5. The melanophore stimulant in pituitary extracts is rapidly inactivated by tryptic but not by peptic digestion; its activity is diminished by boiling with 0·5 % HCl at about the same rate as is that of the uterine stimulant, but much more slowly than that of the pressor substance. The probable identity of the uterine and melanophore stimulants may be inferred.

6. In view of this probable identity of the uterine and melanophore stimulants, the accessibility of the materials, facility of manipulation, specificity and shortness of time implied by the method involving this response, it would seem to be serviceable for testing the activity of posterior lobe extracts in use in the laboratory and hospital.

The experiments were carried out in Prof. MacBride's laboratory at the Imperial College of Science, and the writers were advised on several points by Dr H. H. Dale, F.R.S., to whom grateful acknowledgment is made. The expenses of the research were defrayed by a grant made by the Government Grants Committee of the Royal Society.

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LXIV. ON THE SIGNIFICANCE OF VITAMIN *A* IN THE NUTRITION OF FISH.

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It has recently been shown that the relatively large amounts of vitamin *A* found in the liver of the gadoid fishes is probably derived from their food and ultimately from the marine algae which synthesise this dietary factor. [Coward and Drummond, 1921; Hjort, 1922; Jameson, Drummond and Coward, 1922; Drummond and Zilva, 1922.] That the vitamin *A* probably fulfils some rôle in the development of the young fish is indicated by its presence in the gonads [McCollum and Davis, 1915; Hjort, 1922] and recently it has been shown that the amount stored in the reproductive cells of such a fish as the cod is extraordinarily large [Zilva and Drummond, 1922]. To study the fate of the vitamin in the fish ova it was decided to work with the brown trout which is easy to rear and maintain in the ordinary laboratory.

Vitamin *A* in Trout Eggs.

Feeding tests on rats by the usual procedure demonstrated that trout eggs are very rich in vitamin *A*. A dose of two eggs per day per rat resulted in a resumption of normal growth, and it is to be regretted that tests were not made with smaller doses of the material, but none had been reserved (Fig. 2).

Rearing of Young Trout in the Laboratory.

A thousand fertilised eggs were obtained from the Trout Fisheries, Stirling, and were allowed to hatch out in a single trough which we are describing because it enables trout to be reared with success for considerable periods of time in an ordinary laboratory, and we have encountered many cases where workers have failed to achieve this. All the eggs were placed in a flat boat-shaped receptacle (Fig. 1) 18″ long and cross section 5″ × 5″. The sides are of wood but the ends and bottom are made of narrow-mesh zinc gauze. Such receptacles are placed in an ordinary porcelain sink of which the outlet has been adjusted so as to maintain a depth of half an inch of water whilst a constant stream of water is supplied by means of a pipe leading from the tap. The

incoming stream issues from a glass tube of half-inch diameter and is not directed centrally down the length of the "boat" but at an angle of about 20° to that axis. By this means the water stream does not pass directly out at the far end of the "boat" but tends to form a current round the sides. In this manner the unconsumed food, which must always be borne to the fish down stream, is carried round several times before being washed down the outflow pipe and wastage is thereby prevented.

The sinks and "boats" are kept scrupulously clean and the water current maintained without interruption. Partial shade for the trout is provided by placing a board crosswise over part of the sink. If these precautions are followed it is possible to rear the young trout almost without loss, at any rate for several months.

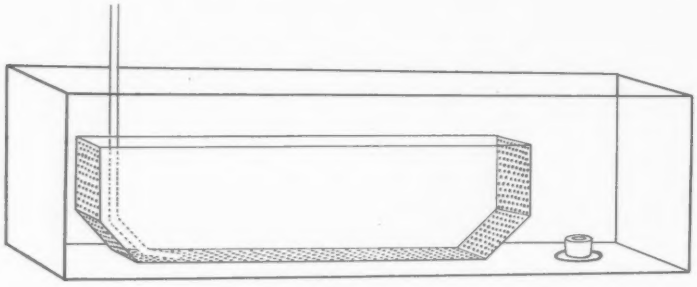


Fig. 1.

Vitamin *A* in Larval Trout.

The ova hatched out with few exceptions and the larvae were allowed to develop without any extra food being given for four weeks. The depth of water was increased to about one inch. It is difficult to obtain information as to the feeding habits of young fresh-water fish, and as far as we are aware there are available no careful studies analogous to Dr Lebour's investigations on marine fish [1918; 1919, 1, 2]. It is commonly believed that larval trout take no food during that stage of their development and that they are purely carnivorous when they begin to feed as soon as the yolk sac is nearly absorbed. Some specimens of young brown trout about one month old from a trout hatchery were examined by us and were found to contain definite remains of partially digested green microscopic plants in their alimentary tracts. It is possible that these were taken in accidentally by the fish or that they were previously ingested by minute crustaceans which the fish had swallowed. The similar presence of marine plants in young fish has been described by Lebour.

About half-way through the larval period our experimental trout were tested again for vitamin *A* and it was found that although the contents of the yolk sac were about half absorbed there yet remained considerable amounts of this dietary factor. At this stage one young fish per day was sufficient

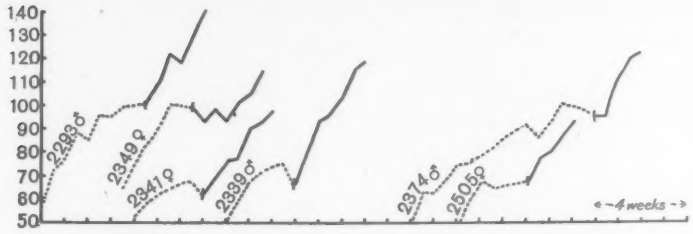


Fig. 2. Fig. 3.

Periods on $-A$ diet only in dotted lines.

Fig. 2. Resumption of growth of rats on feeding two trout eggs previous to basal diet each day.
Fig. 3. Resumption of growth of rats on feeding one larval trout with yolk sac half absorbed.

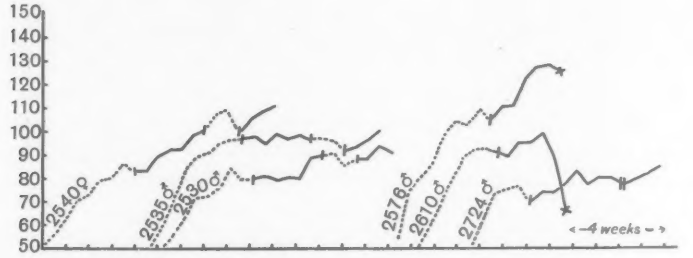
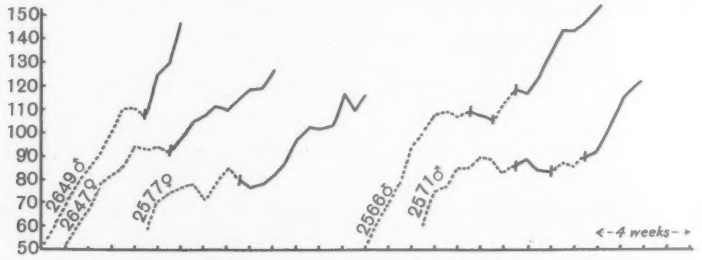


Fig. 4 Fig. 5.

Periods on $-A$ diet in dotted lines.

Fig. 4. Very poor growth of rats on feeding one larval trout with yolk sac completely absorbed.

Fig. 5. Very poor growth of rats on feeding one trout that had been fed on cod muscle from the time of the absorption of yolk sac.



Periods on $-A$ diet only in dotted lines.

Fig. 6. Resumption of growth of rats on feeding one trout that had been fed on liver and yolk of egg from the time of absorption of the yolk sac. Two short periods when the rats refused to eat the trout are seen in curves of rats 2566 and 2571. After a short time on $-A$ only again the rats ate the trout and growth was rapid.

to cause a good resumption of growth in the test rats (Fig. 3). After about four weeks the yolk sacs were practically absorbed in every case and another test was made. From the results it was apparent that the vitamin *A* content of the fish had fallen very greatly and that actual utilisation of that originally present in the yolk sac had occurred (Fig. 4).

The Feeding of Post-larval Trout.

Having found that the supplies of vitamin *A* present in the yolk sac of the newly hatched fish are apparently used up in the larval period, it was important to determine whether such fish are subsequently dependent on their food to maintain their supply of this factor.

Accordingly the remaining fish were divided up into three groups of about 250 each which were treated as follows:

Group I. Diet rich in vitamin *A*; fresh minced rats' liver and ground up yolk of hard boiled egg.

Group II. Diet deficient in vitamin *A*, freshly ground up cod muscle.

Group III. No extra food.

It was found necessary to feed Groups I and II several times a day by slowly dropping the finely minced food pulps into the stream of water in the "boats" so that the small particles were borne down to the fish on the current when the trout would immediately arrange themselves upstream and feed voraciously.

The influence of the feeding on the trout themselves was marked. Those in Group I grew rapidly and were very active, plump and well. Except by an accident late in the experiment when a large number of deaths followed the stoppage of the current of water, there was hardly a death in this group.

The fish on the vitamin *A*-deficient diet of cod muscle lived fairly well but did not grow much or show the vigour of those in Group I. Their weaker constitution was demonstrated by the increasing frequency of deaths as the experiment progressed. The group kept without food exhibited little or no growth but few died until about three weeks after the experiment had been in progress when deaths were suddenly very frequent.

Influence of Food on Vitamin *A* Reserves of Post-larval Trout.

The feeding tests on rats demonstrated in a very striking manner the importance of the qualitative composition of the food supply for maintaining the vitamin *A* in the tissues of these fish. Very slight or no growth (Figs. 4 and 5) was given by supplements of the fish of Group III or those fed on the white fish muscle known to be deficient in vitamin *A* [Drummond, 1918].

The fish from Group I, however, served as an excellent source of the vitamin for rats and led to a prompt resumption of growth (Fig. 6).

Confirmation of this significance of the food supply was given by the survivors of Group II whose diet was later supplemented by an addition of

egg-yolk pulp. They themselves immediately began to grow again and, when used as test material for the one rat surviving this test, showed that their tissues had once again become stored with the vitamin *A*. Similarly, those trout which had been previously starved were fed for ten days with egg; and the rats which had been used to test the starved trout were in the meantime fed only on the diet deficient in vitamin *A*. But when the trout now being fed were added to their diet, the rats immediately resumed growth.

The demonstration of the importance of this qualitative aspect of the diet of young fish may be of some interest in connection with the well-known critical period which coincides with the absorption of the yolk sac. Trout hatchers have assured us that this period is one of great uncertainty in the rearing of young trout, and it is possible that our experiments may suggest to them some modification of the usual existing methods of feeding which might lessen the loss which frequently occurs at that time. Some of our results, which are not fully recorded in this paper, tend to show us that mashed liver, the usual dietary of the fish in hatcheries, may not always supply sufficient of the vitamin *A* for their optimum development and well-being. The full absorption of the contents of the yolk sac is an equally critical stage in the development of marine fish. Fabre and Domergue [1900, 1905] found that certain young fish unable to obtain external food at this stage become anaemic and die, but that an addition of microscopical plant organisms to the water enables them to be reared successfully. The application of such observations to the practical question of sea fishery is well pointed out by Hjort [1914] who emphasises the importance of the immediate availability of suitable plankton food for the survival of larval marine fish.

Summary.

1. The ova of the brown trout normally contain relatively large amounts of the vitamin *A*.

2. During the subsequent development of the young larval fish this substance is in some way utilised so that at or shortly after the stage at which the contents of the yolk sac are absorbed the supplies are almost exhausted, so far as our technique enables us to ascertain.

3. It is probable that this fact is one of the reasons why this stage is so critical a period in the development of the young fish.

4. If, in the post-larval period or even before the yolk sac is completely absorbed, the fish are given food rich in vitamin *A* their growth and development are satisfactory and they appear able to store that factor in their tissues.

5. On a diet containing adequate protein but deficient in the factor *A* no such storage occurs and growth is subnormal.

6. These experiments confirm the previous findings, referred to in the text, which show that the stores of vitamin *A* in the tissues of fish can be derived from the food.

We are indebted to Mr Peart, manager of the Chorley Wood Trout Hatcheries, for invaluable advice in the rearing of the trout, and also to the Medical Research Council for a financial grant which enabled the experiments to be made.

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LXV. NOTE ON KNOOP'S TEST FOR HISTIDINE.

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In performing this test Knoop [1908] adds bromine water to the solution to be tested until there is just a very slight excess. On heating, the excess bromine disappears and in the presence of histidine a brownish red colour is developed; if the latter is in sufficient concentration a dark precipitate settles out. The solution to be tested should be slightly acid. The only substance other than histidine found to give this test is iminazolylethylamine or histamine. The test, according to Knoop, is given with histidine at a dilution of 1 : 1000.

When using this test the writer found that different intensities of colour were produced from solutions of the same concentration of histidine. This peculiar behaviour was accounted for by slight variations in the bromine excess. The colour produced is very soluble in excess of bromine and in dilute solutions of histidine an initial excess may prevent all colour development. The difficulty of adding just sufficient bromine especially to slightly coloured solutions has been overcome by adding a definite excess followed by washing the solution repeatedly with chloroform in a small separating funnel until the chloroform is no longer coloured. By this procedure even strongly coloured meat extracts and other fluids may be successfully tested by Knoop's method. The excess of bromine appears to oxidise the colouring matters and after washing with chloroform such solutions are almost colourless, so that on heating the brown colour due to histidine is readily detected. Most uniform results have been obtained when the washed solution is transferred to a test tube and set in a boiling water-bath.

That the chloroform does not extract any of the histidine compound was shown by adding to a series of test tubes equal amounts of 0·1 % histidine monohydrochloride and adding increasing small amounts of bromine water from a pipette until definite excess of bromine was present. By heating all the test tubes together on the water-bath it was found that there was first a rise, then a fall in colour values corresponding with insufficiency or excess of bromine. The maximum thus obtained was less than that obtained by the chloroform method.

The maximum colour appears to be developed when the histidine has absorbed just three atoms of bromine per molecule. A dilute solution of bromine water was standardised against $N/100$ sodium thiosulphate using a mixture of potassium iodide and starch as indicator on a tile. The bromine solution was 0·00785 N.

In one burette was placed 0·1 % solution of histidine monohydrochloride and in another the standard bromine water just before using it. At ordinary temperature it was found that 10 cc. of the histidine solution absorbed 18 cc. of the standard bromine water. When the solution was kept cold in ice the same quantity of histidine absorbed 16 cc. of the bromine solution, whilst at about 40° in a water-bath it absorbed 21·8 cc. of the bromine solution. (These amounts are not quite definite as on long standing more bromine is absorbed.)

The histidine-bromine solution was in each case transferred to a 100 cc. measuring flask which was filled up to the mark with water, so that the solution represented 0·01 % original histidine. About 10 cc. of each were put into three dry test tubes and heated on the water-bath. The colour developed was maximum when the bromine was absorbed at ordinary temperature.

It follows from this that one molecule of histidine monohydrochloride absorbs approximately three atoms of bromine at ordinary temperature.

To narrow the limits still further five lots of 5 cc. of 0·1 % histidine solution were taken and to these respectively were added 8, 8·5, 9, 9·5 and 10 cc. of standard bromine solution. On diluting to 50 cc. and heating portions, the colour was greatest where 9 cc. had been used. This is the same as the above. The maximum colour developed here was not greater than that got from solutions of the same concentration washed with chloroform.

By the modification above suggested Knoop's test can be obtained with certainty in solutions of histidine at a dilution of 1 : 10,000; a faint colour in pure solutions is still observable at 1 : 20,000.

The colour developed on heating is very variable according to the reaction of the liquid. In markedly acid solution it is a dull yellow brown. In approximately neutral solution, the shade is dark brown. In solutions made faintly alkaline with sodium hydroxide—after bromine has been added and the excess removed with chloroform—a bright pink colour develops which is less stable than that in neutral or acid solutions. If made very faintly alkaline with ammonia instead of caustic soda, a deep purple colour rapidly develops on heating. The presence of ammonia in just the requisite amount gives a more intense colour than in the other cases, but since it is difficult to regulate this amount, and further, since there is reason to believe that the specific value of the test may suffer—for carnosine gives a faint yellow by this treatment—the use of alkali cannot be recommended.

The colour developed from tryptophan by addition of bromine is readily distinguished from the colour due to histidine. The former develops in the cold and is easily extractable with amyl alcohol. The coloured substance in

Knoop's test is not extracted by any of the ordinary solvents and is peculiarly unaffected by reagents other than bromine.

SUMMARY.

By a modification of Knoop's test for histidine its delicacy is increased at least ten times and the test can be performed with greater certainty especially in coloured fluids. The proportion of three atoms of bromine per molecule of histidine is found to give the maximum colour on heating.

REFERENCE.

Knoop (1908). *Beiträge*, **11**, 356.

LXVI. THE ESTIMATION OF CARNOSINE IN MUSCLE EXTRACT—A CRITICAL STUDY.

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In a recent communication [Hunter, 1921] a colorimetric method for the estimation of carnosine in muscle extract was generally outlined. This was based on a method of estimation of iminazoles devised by Koessler and Hanke [1919, 1]. This method depended on the coupling of carnosine with diazotised sulphanilic acid in alkaline solution to form an azo dye. The colour produced was measured against a standard solution of a mixture of Congo red and methyl-orange. Pure solutions of carnosine were found to react very satisfactorily, and in different extracts of the same muscles the colour values were consistent. The accuracy of the method could not be doubted, but the certainty that carnosine was responsible for the total colour production was called in question.

The problem—of the extent to which carnosine is responsible for the production of the azo colour in muscle extracts—may be attacked in two ways: 1. By the elimination of all interfering substances. 2. By a direct method of confirming the carnosine content.

I. The Elimination of Interfering Substances.

Potentially interfering substances. It is well known that aromatic amines and phenols readily couple with a diazotised aromatic amine and are thus potentially interfering substances with the reagent here employed for the estimation of carnosine.

The iminazoles form another well defined group—of which carnosine is only one member—giving the diazo reaction.

The purines which contain the iminazole ring are generally described as substances giving a positive diazo test—this applies at least to the members adenine, hypoxanthine, and xanthine [*v.* Plimmer, 1918].

There are various other substances diversely referred to throughout biochemical literature as giving the diazo test. Of these may be mentioned thymine [Thierfelder, 1908], bilirubin [Neubauer-Huppert, 1913], urochro-

mogen and urobilinogen (Neubauer-Huppert), besides the "neutral-sulphur" compounds of urine (Neubauer-Huppert).

An exhaustive investigation into the behaviour of all substances—confined even to biochemical literature—towards the diazo reagent would probably be endless as well as fruitless. Attention will thus be confined only to those reagents ordinarily employed in the preparation of muscle aqueous extracts and to substances that may be present with a fair degree of probability in the extracts.

A. *Reagents.* Soluble chlorides, sulphates, nitrates, phosphates and sodium acetate, have been tested in relatively high concentrations and found to have no effect on the diazo reagent.

Ammonia and its salts not only interfere with the production of colour from other substances such as histidine [Koessler and Hanke, 1919, 2] but when added alone to the diazo reagent give a yellow colour.

Soluble sulphides give a colour in low concentration. The presence of sulphur in cystine probably accounts for the similar behaviour of that substance towards the diazo reagent.

Ethyl alcohol was found to give no colour with the reagent though Koessler and Hanke find that it inhibits colour production.

Hydrogen peroxide, formaldehyde and acetone all give yellow colours.

Tannic acid in very low concentration gives a marked colour. This is accounted for by its phenolic constituents.

All solutions in which carnosine is to be estimated by means of the diazo reagent should thus be free from ammonium salts, sulphides, and tannic acid. Formaldehyde or phenols, such as thymol, should not be used as preservatives in this connection. Muscle tissue may be preserved in alcohol and extracts by a layer of toluene.

B. *Muscle constituents.* Certain normal muscle constituents which may possibly act as interfering substances in the extracts require a more detailed examination, as these are much less within the control of the worker than substances which may be used in the preparation of the extracts.

The presence of bilirubin, urochromogen and urobilinogen in muscle extracts is too unlikely to claim further consideration for those substances. Thymine may also be dismissed on account of the difficulty with which it is liberated by hydrolysis from nucleic acid [*v.* Jones, 1920].

(1) *Phenols and aromatic amines.* The presence of these substances in protein-free extracts has not been shown by any of the tests used to detect them. Extracts from several types of muscle were shaken in acid solution with ether. The ether extracts were evaporated to dryness and the slight residues taken up in small quantities of water. These gave no diazo reaction nor has Millon's test been found positive either in the extracts themselves or in the ether fractions.

It is known that aromatic amines couple with the diazo reagent in acid solutions, whereas iminazoles and phenols require a weak alkaline medium before

a colour is produced. Thus if a little 1. 2. 4-diaminotoluidine is added to the reagent without the addition of sodium carbonate, a strong orange colour is developed. If an iminazole or phenol is added under the same conditions no colour is produced[1].

Various extracts have been tested with the acid reagent but all with negative results. It may thus be concluded that there are no aromatic amines contributing to the azo colour developed in meat extracts[2].

(2) *Tyrosine.* This substance, which is not extractable by ether, has received considerable attention from the point of view of the diazo reaction. Totani [1915] devised a method to distinguish the azo colour developed by histidine from that developed by tyrosine. The colours were first reduced with zinc and hydrochloric acid, twice the volume of 25 % ammonia added and in both cases a golden yellow colour was obtained. In the case of histidine the addition of hydrogen peroxide changed the colour to lemon yellow whereas the colour was destroyed in the case of tyrosine. A dilution greater than 1 : 20,000 histidine was necessary to get the characteristic final colour.

Among the various substances considered by Totani no mention is made of iminazoles other than histidine, nor of purines, nor of aromatic amines nor of phenols other than tyrosine.

On repetition of Totani's procedure with histidine alongside carnosine, the two substances went through approximately the same colour changes. The final colour was not destroyed and so the presence of tyrosine could not be detected by this method.

Tyrosine gives a positive Millon's test; histidine and carnosine are negative to Millon's. Tyrosine gives a faint Millon's test at a dilution of 1 : 25,000.

A still more delicate test for tyrosine—on which is also founded a method of estimation—has recently been devised by Hanke and Koessler [1922]. This depends on a further modification of the diazo reaction and is approximately as delicate as that reaction is for carnosine. For quantitative purposes the procedure is the same as that adopted for iminazoles and phenols. The test cylinder is allowed to stand for exactly $5\frac{1}{2}$ minutes after the tyrosine solution has been added to the alkaline reagent. This gives rise to a primary yellow colour the intensity of which is not directly proportional to the amount of tyrosine used.

2 cc. of 3*N* sodium hydroxide solution are now added and the contents of the cylinder mixed. This gives rise to a colour intensification with a change of tint towards the red.

One minute after the addition of the sodium hydroxide 0·10 cc. of a 20 % solution of hydroxylamine hydrochloride is added and rapidly mixed. After

[1] The aromatic amines also give a colour in alkaline solutions.

[2] This test would also eliminate bilirubin which gives a blue colour in acid solution [Ehrlich, quoted by Neubauer-Huppert].

a latent period of 5 to 10 seconds an intense bluish red colour rapidly develops. This colour is stable and is directly proportional to the amount of tyrosine present.

Hanke and Koessler note that this intensification is also given by substances capable of a keto-enol tautomerism such as acetaldehyde, acetone and aceto-acetic acid.

It was decided to test this new modification on muscle extracts. It was first observed that the addition of sodium hydroxide and hydroxylamine hydrochloride in the manner above described to the azo colour developed by histidine or carnosine had the effect merely of a proportional dilution. Thus a solution of carnosine gave by the ordinary method a reading of 22·5 mm.; by the new method a reading of 18·3 mm., *i.e.* approximately the same as would be obtained by adding 2·1 cc. water to the cylinder. The following results were obtained on the extracts.

	Reading with diazo reagent mm.	Reading with diazo reagent, NaOH and NH_2OH . HCl mm.	Calculated reading—assuming proportional dilution mm.	Intensification %
Ox muscle	27·2	21·4	21·6	None
Cat „	18·0	16·5	14·3	15·4
Rabbit muscle	10·8	11·3	8·5	33·0
Otter „	15·5	18·5	12·3	50·4
Salmon „	13·7	21·5	10·8	100·0

The percentage of intensification of these extracts is in the order of the yellowness of their azo colours. Thus ox muscle extracts give a colour which matches the carnosine colour standard. The colour developed from cat muscle extract is generally slightly more yellow; that from rabbit almost matches the histidine colour standard; that from otter is still more yellow and that from salmon is so yellow as to be almost unmatchable.

The intensification colours were pink. They did not show the purplish tinge given by tyrosine. No Millon's reaction was obtained in concentrated extracts of salmon muscle and it is concluded that tyrosine is not responsible for the intensification obtained in that tissue.

The specific cause of this intensification has not been determined.

(3) *Purines.* These are normally present in muscle extracts so that a consideration of the individual members is rendered necessary. Adenine and guanine were prepared from commercial plant nucleic acid. This was hydrolysed by suspending in methyl alcohol and passing dry hydrochloric acid gas according to the method employed by Levene [1921] for animal nucleic acid. This process was found to work very satisfactorily. The precipitated chlorides of adenine and guanine were filtered off and separated according to methods described by Jones [1920].

Hypoxanthine nitrate and xanthine were prepared from parts of the adenine and guanine respectively by deaminisation and subsequent purification.

Cytosine and uracil were also prepared from the residues freed from methyl alcohol by further hydrolysis in the autoclave at 160° with 25 % sulphuric acid for five hours according to Jones [1920].

Neither of these pyrimidines gave a colour with the diazo reagent.

0·05 % solutions were made of adenine sulphate, of guanine chloride, of hypoxanthine nitrate and of xanthine—all in 1·1 % sodium carbonate solutions.

1 cc. of each of these was tested in the ordinary way. Guanine and xanthine gave marked colours. The adenine solution showed only a slight reaction and there was no colour in the case of hypoxanthine. With a tenth of the above amounts guanine and xanthine were still strongly positive whilst adenine and hypoxanthine were entirely negative. The intensities of the colour were approximately the same in like concentrations of guanine and xanthine. With 0·025 mg. of guanine hydrochloride in the cylinder a reading of 8·5 mm. was obtained with the histidine colour standard. With 0·05 mg. the reading was 13 mm. Xanthine in the same amounts gave the respective readings 8 mm. and 12·5 mm.[1] The colour production is not directly proportional to the amounts of guanine and xanthine in the cylinder; nor do guanine and xanthine, mixed with a known amount of carnosine, give proportional colours. Thus 1 cc. of a mixture of 1 cc. 18·6 mm. per cc. carnosine solution with 1 cc. 13·5 mm. per cc. guanine solution gave a reading of 13·2 mm. with the test cylinder set at 20 mm. This is 2·8 mm. short of the calculated reading of 16 mm. A similar result was found with xanthine. Adenine and hypoxanthine do not give a colour or inhibit colour production at this concentration, but they tend to make the colour due to carnosine too yellow. Thus with 1 cc. of a mixture of carnosine solution of the above concentration with 1 cc. 0·05 mg. per cc. of adenine sulphate solution, the calculated reading of 9·3 mm. was obtained. Hypoxanthine behaves similarly.

Guanine and xanthine are about half as sensitive towards the diazo reagent as carnosine. If present in appreciable amounts in muscle extract they must seriously affect the estimation of carnosine.

Uric acid in excess gives a slight yellowness to the diazo reagent.

To what extent are purines likely to interfere with the estimation of carnosine in muscle extracts? On the assumption that there is present in extract of meat free purine nitrogen to the extent of 0·045 % as quoted by Lusk [1921] it is unlikely that more than 0·020 % nitrogen represents purines that give a colour with the diazo reagent. If the nitrogen be taken as representing 42 % of the purine molecule—an average figure from adenine, guanine, hypoxanthine, xanthine and uric acid—the purines affecting the diazo reagent amount to about 0·05 %. In muscles with a carnosine content of less than, say 0·1 % the presence of such a proportion of purines would make the results worthless.

[1] The readings are only approximate as the colours are much more yellow than the standard.

To determine the actual purine interference under the conditions of extraction previously recommended, it was then considered necessary to carry out some quantitative fractionations[1].

A quantity of ox muscle was extracted at 70° with small amounts of water until the filtrate no longer gave a positive diazo test. The proteins were precipitated with excess of lead acetate and the excess of lead was removed with disodium phosphate. The filtrate was neutralised with sodium hydroxide and its colour value measured with the carnosine colour standard. This was found to be 1050 mm. per cc.

50 cc. of the extract were taken in a small beaker, made just acid with nitric acid, and silver nitrate was added till a drop of the solution on a tile gave a brown colour with baryta. The test drops were washed back into the beaker.

After settling, the contents of the beaker were filtered by suction through washed asbestos in a small Hirsch funnel. The precipitate was carefully washed with water and after sucking dry was transferred with the asbestos to a small beaker, stirred up with a little water and hydrogen sulphide passed to precipitate the silver. The contents were again filtered through asbestos in the same way and the hydrogen sulphide removed by prolonged aeration with the help of a pump. The filtrate was neutralised. This is the purine fraction.

To the filtrate from the purine fraction excess of solid finely ground baryta was added. The precipitate was allowed to settle, filtered as above, and washed with saturated baryta water. The silver was removed with hydrogen sulphide and the barium with slight excess of sulphuric acid. The slightly acid solution was aerated to remove the hydrogen sulphide and the solution was then neutralised with sodium hydroxide. This is the carnosine fraction.

The filtrate from the carnosine fraction was freed from barium and hydrogen sulphide and neutralised as above. This is the final filtrate.

The exact volume of each of the three fractions was noted. The colour value per cc. of each of the fractions was then measured and their total colour values calculated. The sum total colour value was then obtained and compared with the original, *i.e.* 50 × 1050 or 52,500 mm.

[1] All the carnosine can be extracted from muscle with much smaller proportions of water than recommended by the writer in his previous paper on this subject. A filtrate of about 50 cc. per g. of muscle used was there recommended mainly because the proteins are easily precipitated by slight acidification with acetic acid and heat under those conditions. In concentrated extracts this simple process is not effective and the use of lead acetate or other precipitant is necessitated. The number of washings, however, should not be reduced. There is no need to reduce the proportion of water to muscle when the extract is to be tested only for colour value unless the carnosine content is less than 0·1 %. With that amount in muscle a reading of 12 mm. will still be obtained with the 50 cc. per 1 g. proportion. But for precipitation purposes and other tests it is desirable to keep the filtrate small rather than have to evaporate it to a low volume.

The distribution of colour value is shown by the total colour values in mm. of the three fractions:

Purine fraction	...	1,560
Carnosine fraction	...	47,587
Final filtrate ...	...	2,200
Sum total ...	...	51,347

The unrecovered colour value is thus 1153 mm. or approximately 2 %. The purine fraction accounts for about 3 % and the final filtrate for about 4·5 %. The carnosine fraction represents almost 91 % of the original colour value.

The relatively small error due to purines by the gentle method of extraction recommended is further seen in the table to follow.

Three portions were taken from the same muscle and two extracted as above. The third was extracted at a temperature just under boiling point.

Aliquot portions of the filtrates from the lead precipitates were taken for total nitrogen estimation and for further fractionation into purine and carnosine fractions. The final filtrates were rejected without evaluation. The total nitrogen was also estimated in the carnosine fractions. In the table carnosine, nitrogen and purine (calculated as carnosine) are shown as percentages of the original muscle. The colour values of the purine fraction are at best only a rough estimate on account of the difficulty in comparing with a much redder standard. The error due to the purines in ox muscle is however small.

Meat No.	Carnosine from lead acetate filtrate	Total N from lead acetate fraction	Carnosine from carnosine fraction	Total N from carnosine fraction	Colour value in purine fraction	Difference in % carnosine in lead acetate filtrate and in carnosine fraction
1	0·517	0·530	0·498	0·246	0·004	0·019
2	0·512	0·546	0·495	0·230	0·012	0·017
3	0·533	0·653	0·487	0·258	0·014	0·046

Before considering other substances likely to interfere with the estimation it may be noted that the more drastic extraction process of muscle No. 3 results in a higher percentage colour value. The total nitrogen in this extract is also raised. In the carnosine fraction the percentage falls again into line with the other two samples. The loss is not accounted for by a higher purine value. Though the colour value of the lead acetate filtrate is raised by the more rigorous extraction, the actual percentage of carnosine is lowered. The lowering of colour value becomes obvious even in the lead acetate filtrate if boiling is prolonged. Thus two samples of muscle treated in the usual way showed an average of 0·639 % carnosine in the lead acetate filtrate, whilst three samples of the same muscle after boiling for about 30 minutes showed an average value of only 0·614 % or a fall of 0·025 % reckoned as carnosine.

In muscles 1 and 2 approximately 96 % of the colour is recovered in the carnosine fraction. Calculated as carnosine this accounts for about 50 % of

the nitrogen in the same fraction. There is no reason to doubt that carnosine is responsible for all the colour production in the carnosine fraction from ox muscle. In the case of rabbit muscle and notably salmon muscle, fractionation does not appreciably improve the match. Thus two portions of a rabbit muscle showed from the lead acetate filtrate colour values of 0·092 % and 0·099 % reckoned as carnosine. The carnosine fraction from the same muscles showed each a value of 0·072 % as carnosine. In the latter case the azo colour developed was still very yellow. The colour from salmon muscle extract is slightly more red in the carnosine fraction than in the lead acetate filtrate, yet it is far from satisfactory.

The precipitation process occupies much time and to obtain reliable quantitative results from it, great care must be exercised. With the additional possibility that the carnosine fraction may still contain other colour-giving substances, it would appear of little use to attempt the elimination of interfering substances by the silver-baryta precipitation except in very special cases.

(4) *Iminazoles.* Of this group only histidine and carnosine are known with certainty to occur in animal tissue. Histamine has been found in the intestinal mucosa by Barger and Dale where its presence is attributed to bacterial action [Barger, 1914]. Its isolation from the pituitary gland by Abel is accounted for by Hanke and Koessler through Abel's use of a commercial product. Hanke and Koessler [1920, 2] show that it is absent from fresh hypophysis cerebri.

In protein-free extract from fresh muscle there is no evidence that histidine is present provided the extraction process has been gentle. The ease with which carnosine may be hydrolysed (*v.* later), along with other factors to be considered, renders it important that there should be some ready means of detecting histidine in the presence of carnosine.

As far as the writer is aware there is no known method of separating these substances. They both precipitate in the carnosine fraction in the "arginine separation." Though histidine is more readily precipitated with ammonium-silver than carnosine, yet the latter also precipitates in such relatively dilute solutions as 0·5 % carnosine. Nor could any test be found in the literature to distinguish the two substances.

Various colour tests for histidine were performed on solutions of carnosine. The main differences were found in their behaviour towards the biuret and Knoop tests.

The biuret reaction was found to be entirely negative even in about 30 % solution of carnosine and after standing at least one hour. This difference in behaviour is however of little value for discrimination purposes as relatively concentrated solutions of histidine must be used to get a positive biuret. Thus 1 % solution of histidine monohydrochloride gives the reaction in the cold only after standing for about $1\frac{1}{2}$ hours.

The writer has found Knoop's test to be the most useful way of detecting histidine in the presence of carnosine. Carnosine is quite negative to Knoop's

test. By a modification of the test as explained in the preceding paper, the writer has been able to increase both its delicacy and certainty as a test for histidine. The presence of histidine may be detected with certainty at a dilution of 1 : 10,000.

Though this reaction has not yet the delicacy that might be desired, its very specific nature makes it valuable for work of this kind. Knoop [1908] states that the reaction is also positive for histamine. No other substance, as far as the writer is aware, gives the reaction.

Certain muscle extracts suspected of histidine, were then tested by the modified Knoop's test. Extracts of rabbit and salmon which give notably yellow colours were tested in sufficient concentration but without giving an indubitable Knoop's test. It is thus concluded that histidine is not a factor in causing the yellow colour of those extracts.

In various cases of purchased butcher's meat a positive Knoop's test has been obtained. If the meat is allowed to become just noticeably putrid a very marked reaction is given. Histamine may in part be responsible for the positive test in such cases.

The test may perhaps be used to most advantage as an indirect test for carnosine where the presence of that substance is doubtful in any tissue or fluid. If a protein-free extract is at first negative to Knoop's test, and after hydrolysis (*v.* later) is positive to the test, it appears necessarily to follow that the unhydrolysed liquid contains carnosine.

The test can also be applied to elucidate some other problems that had arisen in the course of this work. The effect of heat on carnosine solutions will first be considered.

The effect of heat on solutions of carnosine.

In the writer's preliminary communication on the estimation of carnosine, a slight fall was noted in the colour values of solutions of carnosine heated on the water-bath for one hour. Similar experiments have since been repeated but the period of heating has been extended to four hours. Thus a number of solutions of carnosine nitrate with an original colour value of 22 mm. per cc. showed after four hours on a boiling water-bath an average value of 19·4 mm. per cc. or a fall of 2·6 mm. per cc.

10 cc. of a 0·5 % solution of carnosine as nitrate was brought to dryness three times in an open basin on a steam-bath. The total period of heating was about four hours. It was finally taken up in about 2 cc. of water and Knoop's test applied. A decided brown colour was developed indicating that in the process the carnosine had been partly hydrolysed, giving rise to histidine. This observation confirms the fall in colour value.

Though four hours is a relatively long period of heating, the fact that carnosine is thus destroyed in pure neutral solution indicates that care should be exercised in the process of extraction from muscle.

The fall in colour value as estimated in carnosine from 0·639 % to 0·614 %

on prolonged extraction of the muscle, previously noted (p. 646), may be accounted for by the destruction of carnosine. The occurrence of histidine in muscle extracts will thus arise more likely from the hydrolysis of carnosine than from the hydrolysis of histidine-containing proteins. For if the latter process occurred to a marked degree the colour values would tend to rise rather than to fall.

It will thus be observed that any process of evaporation at atmospheric pressure must result in at least a partial destruction of the substance.

Solutions of carnosine whether pure or in muscle extracts may be evaporated *in vacuo* to dryness with very little destruction. Thus 10 cc. of a neutral dilute meat extract were evaporated to dryness at 20 mm. pressure of mercury from a 200 cc. distilling flask on a water-bath about 60°. Exactly 10 cc. of water were then pipetted into the flask and rinsed round to dissolve the dried residue. The colour value of the original solution was 25·6 mm. per cc. whilst that of the dried substance taken up in 10 cc. of water was 25 mm. per cc. (The volume of solid in the original 10 cc. was regarded as negligible.) Repetition of this experiment still showed a loss in colour value of about 2 %.

This experiment at the same time goes to show that a very small proportion, if any, of the colour-producing substances in muscle extract is volatile. It serves to confirm the results obtained by ether extraction and Millon's test.

(5) *Other substances.* The effects of cystine, leucine and arginine on the diazo reagent have been measured by Hanke and Koessler [1920, 1]. These workers find that there is no interference so long as the ratio of cystine to histidine does not exceed 6 : 1, which is higher, they note, than has yet been found in any protein. Cystine may thus be dismissed as a probable interfering factor in muscle extracts.

The effects of arginine and leucine are less marked than those of cystine.

Creatine in large amounts gives a slight yellowness to the reagent.

Creatinine, urea and lactic acid have been found to have no effect on the reagent.

Such a process of elimination as has here been attempted must yield mainly negative conclusions. None of the substances most likely to interfere with the colour values has any very marked effect. In the case of ox muscle it has been shown that most probably less than 5 % of the colour value is not due to carnosine. In cat and frog extracts, judging merely from the colour development, the non-carnosine colour value is likely to lie within the same limit, but of salmon little can be said except that it contains carnosine. Any elimination method must necessarily be unsatisfactory unless the substance can be obtained pure; and many factors militate against that possibility in the case of carnosine. Among those may be noted the high solubility of the substance in water, its facility for adsorption, the inadequacy of the methods for its fractional precipitation and the ease with which it is hydrolysed. It is upon this last property that the writer has sought a more direct method for confirming the values.

II. Hydrolysis Experiments.

(1) *The stability of histidine.* Histidine has been observed by Koessler and Hanke [1919, 2] to be remarkably stable towards hot concentrated hydrochloric acid. They observed that when 2 cc. of a 1 % solution of histidine dihydrochloride were heated on a boiling water-bath for 10 hours with 25 cc. concentrated hydrochloric acid the histidine was quite unchanged, as shown by the colour values recovered. Over a shorter interval the writer has confirmed those findings as shown by the following experiment.

Into each of five numbered test tubes there was introduced 1 cc. of a solution of histidine along with 3 cc. concentrated hydrochloric acid. These were then set on a boiling water-bath and one removed every 15 minutes. As each test tube was removed from the water-bath it was cooled, about 1 sq. mm. of litmus paper introduced, and the contents neutralised with sodium hydroxide. The contents were again cooled and poured into a 25 cc. flask. The test tube was repeatedly washed into the measuring flask, then water was added up to the mark. 1 cc. of this was taken for measuring the colour value against the histidine colour standard. The original value of 23 mm. was obtained by directly diluting 1 cc. of the original to 25 cc. and taking 1 cc. for the test. The colour values after periods of boiling lasting 15, 30, 45, 60 and 75 minutes were found respectively to be 23·2, 23·1, 23, 22·9 and 23 mm. High concentrations of sodium chloride affect neither the tint nor the intensity of the colour developed.

(2) *Hydrolysis of carnosine—theoretical considerations.* It thus seemed very probable that if carnosine were hydrolysed with hydrochloric acid, the colour value would continue to fall until the reaction was complete. If the diazo value of the solution were measured before hydrolysis and again after hydrolysis, the ratio of the colour values thus obtained should be that of their molecular colour values. The molecular colour value of histidine was previously found to be 117 million mm. as compared with 114 million mm. determined by Koessler and Hanke. A revised determination of the molecular colour value of carnosine has led the writer—mainly from increased skill in matching the histidine and carnosine colour standards—to the conclusion that the molecular colour value of carnosine is somewhat greater than the 152 million mm. previously published, viz. 161 million mm. This value is checked by the following considerations.

The histidine colour standard is a mixture of 0·22 cc. methyl-orange with 0·20 cc. Congo red in 100 cc. water. For convenience call this S_H.

The carnosine colour standard is a mixture of 0·10 cc. methyl-orange with 0·25 cc. Congo red in 100 cc. water. Call this S_C.

These amounts were taken from 0·1 % stock methyl-orange and from 0·5 % stock Congo red [*v.* Hunter, 1921].

On comparing in the colorimeter it is found that 24 mm. $S_C = 28{\cdot}5$ mm. S_H.

From the table previously published [Hunter, 1921] 0·040 mg. of carnosine in the test cylinder set at 20 mm. showed a reading of 24 mm. S_C.

0·04 mg. of carnosine should thus give a reading of 28·5 mm. S_H.

The molecular colour value of carnosine is thus

$$\frac{28{\cdot}5}{4} \times 226 \times 10^5,$$

or approximately 161 million mm. S_H.

Now, carnosine yields by theory on hydrolysis 68·5 % of histidine, *i.e.* 0·04 mg. of carnosine yields on hydrolysis 0·0274 mg. of histidine.

0·04 mg. of histidine monohydrochloride gives a reading of 22·4 mm. S_H.

[N.B. The molecular colour value of histidine is thus

$$\frac{22{\cdot}4}{4} \times 209 \times 10^5,$$

i.e. approximately 117 million mm. S_H.]

Histidine monohydrochloride contains 74 % histidine, *i.e.* 0·04 mg. of histidine monohydrochloride contains 0·0296 mg. of histidine.

0·01 mg. of histidine will thus give by calculation a reading of $\frac{22{\cdot}4}{2{\cdot}96}$ or 7·57 mm. S_H, and

0·0274 mg. of histidine will give 20·7 mm. S_H.

That is, a solution of carnosine giving an original reading of 28·5 mm. S_H will give after hydrolysis a reading of 20·7 mm. S_H.

The ratio of the final reading to the original reading should be the same as the ratio of the molecular colour value of histidine to the molecular colour value of carnosine.

Thus $$\frac{20{\cdot}7}{28{\cdot}5} = \frac{117}{161} = 0{\cdot}73.$$

For convenience this figure will be termed the *hydrolysis quotient*.

(3) *Experimental.* The results obtained from the actual hydrolysis of carnosine solutions are less satisfactory than one might expect from the above considerations. Thus a series of carnosine solutions were treated in exactly the same way as that described for histidine. 0·1 % solution of carnosine was used. The original value of 1 cc. from 25 cc. dilution was 28·5 mm. S_H. After hydrolysis for periods of 15, 30, 45, 60 and 75 minutes 1 cc. showed the respective colour values 20·4, 19, 18·2, 18·1 and 18 mm. S_H. After 15 minutes' hydrolysis the colour was more yellow than S_H.

Carnosine is thus very readily hydrolysed and under the above conditions part of the histidine is destroyed. After the initial sudden fall from 28·5 mm. to 20·4 mm. continued boiling lowers the value at a much diminished rate. After 30 minutes' boiling the values remain almost constant but the colours are too yellow—indicating some destruction. It would appear that in the course of the hydrolysis the histidine passes through an unstable phase in which the iminazole ring is readily disrupted. The histidine that emerges from that hypothetical intermediate condition resists further boiling.

Other hydrolytic agents, such as sodium hydroxide and acetic acid, besides

different conditions of temperature and concentration were tested, in the attempt to overcome the difficulty. Finally an approximately constant-boiling mixture of hydrochloric acid was used and the hydrolysis conducted at 90°. Under these conditions a strictly quantitative conversion is not yet attained, but a comparison of the results from various extracts under standard conditions would appear to be of some weight. The extracts hydrolysed were of such a concentration that 1 cc. of the final dilution gave approximately the same values. To the amount of extract to be hydrolysed exactly half its volume of concentrated hydrochloric acid was added. The following are the results from a solution of pure carnosine and from muscle extracts of cat, of ox, and of salmon. This ox extract was slightly positive to Knoop's test. All the readings were taken with the histidine standard.

Time mins.	Carnosine mm.	Cat muscle extract mm.	Ox muscle extract mm.	Salmon muscle extract mm.
Original	28·5	29·0	28·5	27·0
15	25·4	24·3	26·0	26·5
30	23·3	23·0	23·5	24·0
45	21·5	21·8	22·7	22·5
60	21·0	21·2	22·0	22·1
75	20·9	20·7	21·7	21·4
Hydrolysis quotients after 75 mins.	0·73	0·71	0·76	0·79

The carnosine solution and the cat muscle extract agree very well in both their rate of fall and in their hydrolysis quotients. The ox muscle quotient is slightly high, probably owing to the presence of either histidine or histamine in the original. The salmon extract quotient is still higher, but it is remarkable in face of the very yellow azo colour given by the original that the rate of fall accords so well with the others.

Although this method of hydrolysis is not sufficiently sensitive to show that a definite percentage of the colour is due to carnosine, it at least gives one a sense of assurance in the use of a very unspecific reagent for the estimation of carnosine in such a complex solution as muscle extract. It would further seem to indicate that carnosine is responsible for a very high percentage of the colour as measured from fresh muscle extracts treated only with lead acetate.

The specific cause of the yellow colour produced in such cases as salmon has not been found. In the case of rabbit muscle, it has been observed that the colour is more red when the carnosine content of the muscle is high and yellow when low. Some cat and frog muscles with a low carnosine content also showed a yellow colour. This is not surprising as the chances of interference are greatly increased when the test portions are less dilute.

Though purines certainly give rise to a small error and exact quantitative results cannot be got when the colour is not of the right tint, yet the total error in the lead acetate filtrate is too small to eliminate. With a better hydrolytic agent it might yet be possible to evaluate the error.

Carnosine Content of some Muscles.

With the method of extraction and treatment of the extract as previously described, the following results have been obtained. Each result represents the percentage colour value reckoned as carnosine in the fresh skeletal muscle. Further, each result represents the average of at least two results obtained from different pieces of the same muscle.

For convenience the amounts found are given from lowest to highest carnosine contents. The contents for four members of each species of animal are given.

Rabbit muscles	0·026	0·064	0·090	0·101	% carnosine
Frog „	0·107	0·128	0·142	0·280	„ „
Cat „	0·123	0·203	0·336	0·380	„ „
Ox „	0·340	0·400	0·515	0·640	„ „

The results show that the carnosine content of muscle varies not only with the species of animal but varies greatly in different animals of the same species. The highest values obtained in the one species are two to four times greater than the lowest values in the same species. This finding is at variance with that of Clifford [1921, 2]. This worker finds, for example, ox muscle to have an almost constant carnosine content of 1·1 %. But apart from the constancy of the results found, the writer is compelled to question their accuracy. In a previous paper [1921, 1] Clifford finds that 0·02 mg. of histidine gives a value of 10·75 mm. measured with the Koessler and Hanke histidine standard. With these data—and assuming the author means 0·02 mg. of histidine *dihydrochloride*—the molecular colour value of the histidine is

$$\frac{10{\cdot}75}{2} \times 228 \times 10^5,$$

which is approximately 122 million mm. S_H.

With the same colour standard Clifford records that 0·1 mg. of carnosine gives a reading of 30 mm. The molecular colour value of Clifford's carnosine is thus $30 \times 226 \times 10^4$ or approximately 68 million mm. As previously stated, the writer finds the molecular colour value of carnosine against this same colour standard to be 161 million mm. Assuming this figure represents 100 % pure carnosine, the carnosine employed by Clifford is thus only about 43 % pure. The carnosine content of the various muscles tested by Clifford should from this point of view be about 43 % of the values actually given.

Summary.

1. Extracts to be tested for carnosine should be free from ammonium salts, sulphides, phenols and aldehydes.

2. The degree of yellowness of the azo colours developed from muscle extracts is in the order of the percentages of the intensification of the azo colours as given by sodium hydroxide and hydroxylamine.

3. In ox muscle purines are responsible for about 3 % of the colour value reckoned as carnosine. In the same tissue there is probably about other

2 % of the colour not due to carnosine. The error in the method due to colour-producing substances other than carnosine is probably about 5 % in ordinary muscles.

4. Histidine under certain circumstances may be present in muscle extracts.

5. A test has been found to distinguish histidine from carnosine.

6. A more certain means than the diazo reagent is given for the identification of carnosine in any tissue.

7. The fall in colour value of hydrolysed meat extracts agrees with that of pure solutions of carnosine.

8. The carnosine content of muscle varies with the species of animal and with different members of the same species.

The writer is much indebted to Prof. Cathcart for his inspiring guidance throughout this work.

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LXVII. IDENTIFICATION OF INULIN BY A MYCOLOGICAL METHOD.

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In previous publications [1917, 1919, 1920] we have described a general mycological method, theoretically devised by one of us (C.) some years ago in Ceylon, which we have found useful in the identification of various carbohydrates and other carbon compounds. We propose in the present paper to describe briefly how this method can be applied to the determination of inulin.

It is generally stated that there is no organism which induces a complete fermentation of inulin, that is to say, fermentation with production of gas, but one of us (C.) has found a fungus which causes a complete fermentation of this carbohydrate with large production of gas. This fungus is *Monilia macedoniensis* Castellani and allied species, which ferment with production of gas in addition to inulin the following carbohydrates: glucose, levulose, galactose and saccharose.

By means of this fungus in conjunction with certain other fungi, it is possible to identify inulin, using a modification of the general mycological method we described some time ago for the identification of various sugars.

Technique. Let us suppose we have a substance about which we want to decide whether it is inulin or not. A sterile 1 % solution in sugar free peptone water is made and distributed into two tubes, No. 1 and No. 2, each containing a Durham's fermentation tube or similar appliance. The following procedure is then used:

(*a*) No. 1 tube is inoculated with *Monilia macedoniensis* Cast., No. 2 with *Monilia tropicalis* Cast. The two tubes are placed in an incubator at 35–37° for 72 hours. If after that time, No. 1 tube contains gas and No. 2 tube does not, we can come to the conclusion that the substance is inulin. This is easily understood by keeping in mind the fermentative reactions of the two monilias: *Monilia macedoniensis* ferments with production of gas, only the following carbon compounds: glucose, levulose, galactose, saccharose and inulin. *Monilia tropicalis* Cast. ferments with production of gas, only glucose, levulose, maltose, galactose and saccharose.

$$\left.\begin{array}{ll} \textit{Monilia macedoniensis} \text{ Cast.} & + \\ \textit{Monilia tropicalis} \text{ Cast.} & 0 \end{array}\right\} = \text{Inulin.}$$

(*b*) No. 1 tube is inoculated with *Monilia macedoniensis* Cast.; No. 2 with *Monilia rhoi* Cast. The two tubes are placed in an incubator at 35–37° for 72 hours. If after that time No. 1 tube contains gas and No. 2 does not we can come to the conclusion that the substance is inulin. This is easily understood remembering that *Monilia macedoniensis* ferments with production of gas, only glucose, levulose, galactose, saccharose and inulin, and *Monilia rhoi* ferments with production of gas, only glucose, levulose, galactose and saccharose.

Monilia macedoniensis Cast. +
Monilia rhoi Cast. 0
} = Inulin.

(*c*) No. 1 tube is inoculated with *Monilia macedoniensis*; No. 2 with *B. pseudocoli* or *B. neapolitanus*, or any other strain of the *communior* group of *B. coli* (ferment saccharose). The tubes are incubated at 37° for four days. If then tube No. 1 contains gas and tube No. 2 does not, we can again come to the conclusion that the substance is inulin, since glucose, levulose, galactose or saccharose would have been fermented also by *B. pseudocoli* or *B. neapolitanus* or any other strain of the *Coli communior* group.

Monilia macedoniensis Cast. +
B. coli communior (*B. pseudocoli* Cast., *B. neapolitanus* Emmerich, etc.) 0
} = Inulin.

(*d*) No. 1 tube is inoculated with *M. macedoniensis* Cast., No. 2 tube with *B. asiaticus* Cast. The two tubes are placed in an incubator at 37° for four days. If after that time No. 1 tube contains gas and No. 2 does not, we can come to the conclusion that the substance according to all probabilities is inulin. This is easily understood by remembering the fermentative reactions of the two organisms. *M. macedoniensis* ferments only glucose, levulose, galactose, saccharose and inulin with production of gas; whilst glucose, levulose, galactose and saccharose are also fermented by *B. asiaticus*; it must therefore be inulin.

Monilia macedoniensis Cast. +
B. asiaticus Cast. 0
} = Inulin.

Identification of Inulin when present with some of the more common Fermentable Substances.

If we suspect that a liquid contains inulin mixed with some of the more usual fermentable substances such as glucose, levulose, maltose, etc., we can find out the presence of inulin in the following manner. The mixture is fermented with *Monilia tropicalis* Cast. If, after exhaustion with *M. tropicalis*, the liquid can still be fermented with *M. macedoniensis* with production of gas, the inference is that the liquid contained inulin. Of course, the precaution should be taken of selecting strains of *M. tropicalis* and *M. macedoniensis* with approximately equal fermentative power on glucose, levulose, galactose and saccharose, which carbohydrates they both ferment.

Addendum.

For the reader's convenience we annex a table containing the fermentative characters of the various fungi and bacteria we use in our method, and we give also a list of the principal mycological formulae which we have devised and employed in the identification of various sugars and other carbon compounds. It is essential to use strains with permanent biochemical reactions. Acid fermentation without production of gas is not taken into account.

Table showing fermentation reactions of certain fungi and bacteria.

	Glucose	Levulose	Maltose	Galactose	Saccharose	Lactose	Mannitol	Dulcitol	Dextrin	Raffinose	Arabinose	Adonitol	Inulin	Sorbitol	Starch	Glycerol	Inositol	Salicine	Amygdalin	Isodulcitol	Erythrytol
Monilia balcanica Cast. ...	G	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M. Krusei Cast.	G	G	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M. pinoyi Cast.	G	G	G	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M. metalondinensis Cast. ...	G	G	G	G	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M. tropicalis Cast.	G	G	G	G	G	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M. rhoi Cast.	G	G	0	G	G	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M. macedoniensis Cast. ...	G	G	0	G	G	0	0	0	0	0	0	0	G	0	0	0	0	0	0	0	0
Bacillus coli Escherich ...	G	G	G	G	0	G	G	G	G	G	G	0	0	G	0	G	0	G	0	G	0
B. pseudocoli Cast.	G	G	G	G	G	G	G	G	G	G	G	0	0	G	0	G	0	G	0	G	0
B. paratyphosus B var. *M* ...	G	G	G	G	0	0	G	G	G	0	G	0	0	G	0	0	G	0	0	G	0
B. paratyphosus A Schottmüller	G	G	G	G	0	0	G	G	G	0	G	0	0	G	0	0	0	0	0	G	0
B. asiaticus Cast.	G	G	G	G	G	0	G	0	G	G	G	0	0	G	0	G	0	0	0	G	0
B. pseudoasiaticus Cast. ...	G	G	G	G	G	0	G	G	G	G	G	0	0	G	0	G	0	G	0	G	0

G = gas; 0 = absence of gas. Simple acid fermentation is not taken into account.

Mycological Formulae.

Inulin.

Monilia macedoniensis Cast. ... ... +
M. tropicalis Cast. ... ... 0 } = Inulin

M. macedoniensis Cast. ... ... +
M. rhoi Cast. ... ... 0 } = Inulin

M. macedoniensis Cast. ... ... +
Bacillus coli communior (*B. pseudocoli*, *B. neapolitanus*) 0 } = Inulin

M. macedoniensis Cast. ... ... +
B. asiaticus Cast. ... ... 0 } = Inulin

Maltose.

M. tropicalis Cast. ... ... +
M. macedoniensis Cast. ... ... 0 } = Maltose

M. metalondinensis Cast. ... ... +
M. macedoniensis Cast. ... ... 0 } = Maltose

M. pinoyi Cast. ... ... +
M. krusei Cast. ... ... 0 } = Maltose

M. pinoyi Cast. ... ... +
M. macedoniensis Cast. ... ... 0 } = Maltose

Galactose.

M. metalondinensis Cast. ... ... +
M. pinoyi Cast. ... ... 0 } = Galactose

M. metalondinensis Cast. ... ... +
M. krusei Cast. ... ... 0 } = Galactose
M. macedoniensis Cast. ... ... +

Galactose (continued).

M. tropicalis Cast.	+	= Galactose
M. bronchialis Cast.	0	
M. tropicalis Cast.	+	= Galactose
M. macedoniensis Cast.	+	
M. krusei Cast.	0	
B. paratyphosus B Schottmüller	+	

Saccharose.

M. tropicalis Cast.	+	= Saccharose
M. metalondinensis Cast.	0	
M. rhoi Cast.	+	= Saccharose
M. pinoyi Cast.	0	
M. tropicalis Cast.	+	= Saccharose
B. coli communis (sensu stricto)	0	
M. tropicalis Cast.	+	= Saccharose
B. paratyphosus B Schottmüller	0	
M. macedoniensis Cast.	+	= Saccharose
B. coli communis (sensu stricto)	0	
B. coli communior	+	
M. macedoniensis Cast.	+	= Saccharose
B. paratyphosus B Schottmüller	0	
B. coli communior	+	
B. coli communis Escherich (*sensu stricto*)	0	= Saccharose
B. neapolitanus Emmerich	+	
B. coli communis Escherich (*sensu stricto*)	0	= Saccharose
B. asiaticus	+	

Levulose.

M. krusei Cast.	+	= Levulose
M. pinoyi Cast.	0	

Glucose.

M. baleanica Cast.	+	= Glucose
M. krusei Cast.	0	

Inositol.

B. paratyphosus B var. *M* Schottmüller	+	= Inositol
B. paratyphosus A Schottmüller	0	

CHEMICO-MYCOLOGICAL FORMULAE.

Saccharose.

Fehling	0	= Saccharose
M. tropicalis Cast.	+	

Lactose.

Fehling	+	= Lactose
B. paratyphosus B Schottmüller	0	
B. coli communis Escherich	+	

Pentose.

Fehling	+	= Pentose (generally arabinose)
M. tropicalis Cast.	0	
B. paratyphosus B Schottmüller	+	
B. coli communis Escherich	+	

+ = gas; 0 = no gas; simple acid fermentation is not taken into account.

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LXVIII. FEEDING EXPERIMENTS IN CONNECTION WITH VITAMINS *A* AND *B*.

III. MILK AND THE GROWTH-PROMOTING VITAMIN.

IV. THE VITAMIN *A* CONTENT OF REFINED COD-LIVER OIL.

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Work carried out at the Research Laboratory, Port Sunlight.

(*Received July 10th, 1922.*)

III.

During 1920 Osborne and Mendel [1920] published a paper entitled "Milk as a Source of Water-soluble Vitamin," in which they state that they are unable to produce results similar to those obtained by Hopkins in his classical experiments published in 1912 [Hopkins, 1912].

The administration of 2 cc. of milk per day in these experiments was sufficient to provide the accessory factors for growing rats, but the American authors did not obtain anything approaching normal growth until at least 16 cc. per day were supplied.

Osborne and Mendel's experiments included the use of winter milk (which was probably deficient in vitamins owing to stall-feeding of the cattle) and also milk from grass-fed animals. In each case comparable results were obtained. The authors also considered the possibility of an unsuspected inorganic substance in Hopkins' salt mixture, but experiments showed that this was not the cause of the discrepancy.

Subsequent to this, Hopkins [1920] repeated his experiments and confirmed his previous findings, without, however, being able to throw any light on the discrepancies of the American investigators. He suggests that a seasonal variation in the food value of milk may be a contributing cause, although an experiment upon goats' milk, secreted on winter and summer diets, planned to elucidate this point, failed to yield much evidence. He also suggested that there was a seasonal variation in the growth energy of rats.

In view of these conflicting results, the experiment now to be described may be of some interest, although the circumstances under which it was carried out were not comparable (as regards age of animals) to those in the experiments of Hopkins and of Osborne and Mendel referred to above.

The chief point of difference was that the animals used in the present work were the survivors from a previous experiment which had already

lasted for 101 days, the results of which showed conclusively that the materials tested were almost entirely deficient in vitamin *A*. The average weight of the animals at the commencement of this experimental period was 57 g. and at the termination 80 g.—an increase of only 23 g. as compared with the normal for this period, which is 142.

There were ten of these survivors and their average age when selected for use in this new experiment was 150 days. Eight of them were showing marked signs of keratomalacia and general lack of condition and the other two were also out of condition but not to the same extent.

They received throughout the experiment 2 cc. of cow's milk per animal per day and the only other food supplied was entirely deficient in vitamins and composed as follows:

Basal dietary	85 %
Steam distilled palm-kernel oil ...	15 %

[For method of preparation see Stammers, 1921.] No antineuritic or anti-scorbutic was given, the milk supplied being the only source of these factors available to the animals.

The experiment was carried on for 111 days, and during this period the animals were weighed twice a week, first thing in the morning, before feeding.

It should be stated that the experiment was carried out during the months of December, January, February and March, and the milk used was obtained from stall-fed cows. Butter-fat obtained from the same source during this period showed the effect of stall feeding in giving growth far below the normal (see graph) when used as a control in other experiments, so that the milk may be considered to have been of fairly poor quality as regards its vitamin content.

Results.

As already mentioned, the average weight of ten animals at the commencement of the experiment was 80 g. and their average age was 150 days. In 111 days the increase in average weight was 38 g., *i.e.* 80–118.

The normal increase for this period and age is 43 g., *i.e.* 202–245; hence there is a deficit of only 5 g. in average weight over a period of nearly sixteen weeks.

The animals improved out of all knowledge in general condition, the keratomalacia disappearing and the only difference from normal animals was in their weight and size. Otherwise, they appeared to be in excellent health.

A graph is appended (Fig. 1) which shows

A, growth from milk,
a, normal for *A*,
B, *C*, growth from winter butter-fat,
b, *c*, normals for *B*, *C*.

It will be noticed that the butter-fat curves are almost super-imposable.

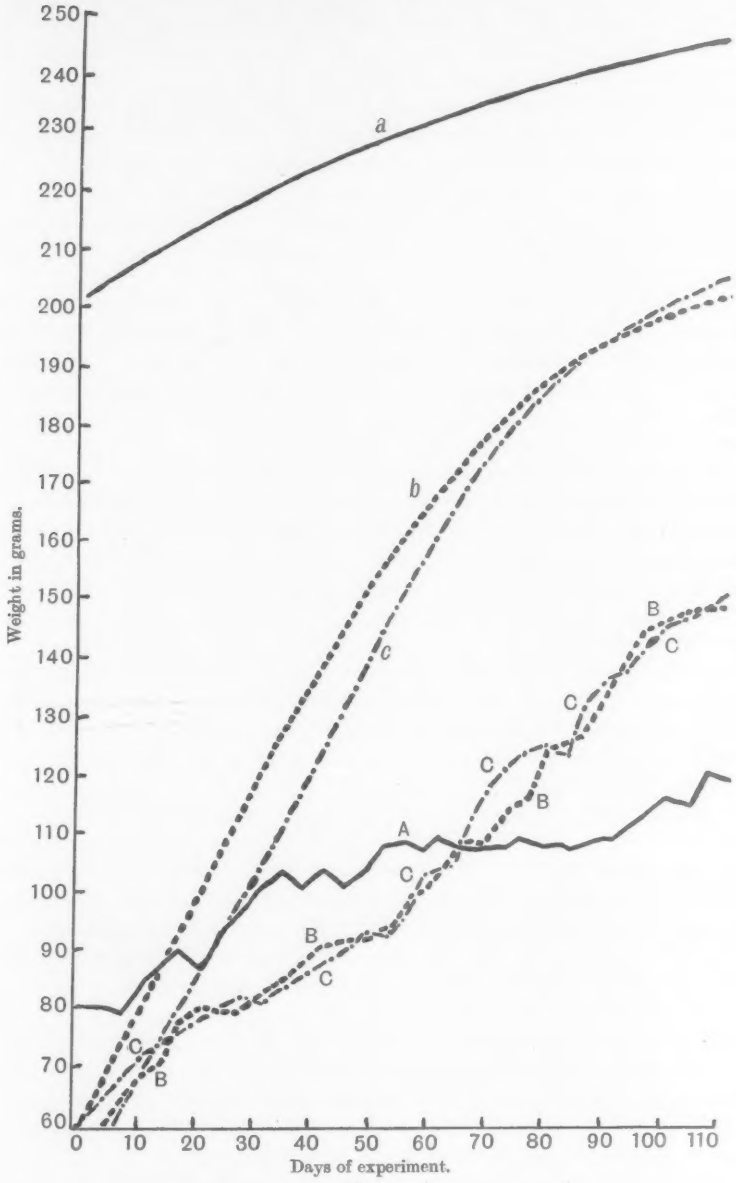

Fig. 1.

a, *b*, *c*, Donaldson's curves of normal growth.
A, growth from milk. B, C, growth from butter-fat.

Conclusions.

While it is not maintained that this experiment either definitely confirms the results obtained by Hopkins, or disproves the statements of Osborne and Mendel, it seems to throw some additional weight of evidence in favour of the former, although, for the reasons already mentioned, the conditions under which the various experiments were carried out are not comparable.

It is considered, however, that the probabilities are that if an attempt had been made to duplicate Hopkins's experiments by making the conditions similar, the results, even with the sample of winter milk used, would have confirmed the findings of this worker.

If young animals had been used, the initial "growth momentum," as it were, derived from the food consumed prior to weaning, plus the 2 cc. of milk administered daily during the experimental period, would probably have sufficed to give normal growth, having regard to the seasonal variation; in other words the growth from milk would have compared favourably, if nothing more, with that from winter butter-fat.

It may be urged that an adult requires less vitamins to keep it in condition than does a young animal and an explanation of the results of this experiment may be found in this fact: on the other hand, this hypothesis is, in the writer's opinion, only applicable in the case of an animal which has received a normal diet up to the age of maturity and not to animals such as those used in this work, which had been fed on a vitamin-deficient diet since they were seven weeks old.

A further point of interest is now touched upon. When an animal has been fed upon a deficient diet and is suddenly placed upon one containing a greater or less quantity of the growth-promoting vitamin, a considerable stimulus is usually applied by the latter to the growth impulse and this can generally be well seen on the graphic record of the growth. It is quite common for animals in such a case to put on a large increase in weight in the first week, but if this occurs there is usually an almost corresponding drop within the next week or so. It will be noticed that, in this case, the effect of any stimulus applied by the administration of the milk was not reflected in a sudden jump in weight; in fact, during the first week there was a drop and if the curve is smoothed out, it will be seen that it remains almost parallel to the normal.

A possible explanation is that the deficiency disease caused by the previous experiment had suppressed the growth impulse to such an extent that the latter could not immediately respond to the stimulus applied. It is also possible that the greater the age of the animal the greater must be the magnitude of the stimulus to produce this effect, even though this may appear, under certain circumstances, to contradict the axiom that the older an animal is, the less vitamin it requires to keep it in health.

A possible criticism may be applied (owing to the advanced age of the animals used) to the conclusions drawn and therefore attention is invited at this stage to the next paper (IV).

It will be observed that in four of the experiments there described, the animals were also of a mature age (varying from 130 to 160 days) and in three of the experiments had been previously fed upon a diet which had failed to maintain adequate growth, thereby approximating to the conditions under which the present experiments were carried out.

Cod-liver oil is well known to be a rich source of vitamin *A*, but in spite of this fact its administration failed to raise the weights of the animals concerned to the normal, but the curves ran roughly parallel to that of normal growth (Donaldson) without any marked approach to the latter.

It might have been expected that cod-liver oil would be sufficiently potent to overcome the stagnation of the growth impulse and raise the weights to somewhere near the normal, but this did not take place even in any individual rat.

It therefore seems that age is the important factor and that once an animal has reached a certain age, either (*a*) the amount of vitamin which would have been adequate to give normal growth in a young animal, is insufficient, or (*b*) no amount of vitamin can fully restore the growth impulse.

The writer's opinion is that the latter is probably the correct explanation.

A study of these results therefore would seem to indicate that the curve of normal growth remains constant, within limits, for age and not for weight, and, if for any reason the growth impulse has been inhibited, an attempt at restoration of the impulse by however powerful a stimulus cannot succeed to an extent greater than that which would be expected from the age of the animal.

IV.

Six experiments are described in this paper and two varieties of cod-liver oil were used. It has already been established that crude cod-liver oil is exceedingly rich in vitamin *A* [Zilva and Drummond, 1921]. Of the two varieties used in the experiments now described, one, *A*, was an ordinary sample procured from a pharmacist and of a type commonly supplied for medicinal purposes; the other, *B*, was more crude and a brand suitable for administration to cattle. Sample *A* was used in Exps. 1, 2, 4, 5, and 6, and sample *B* in Exp. 3.

The animals in Exp. 1 were 160 days old at the commencement and had been fed (for the previous 110 days) upon butter-fat and used as a control in another set of experiments. Their average weight was 148 g. Those in Exp. 2 were also 160 days old and had previously been fed (also for 110 days) upon a partially deficient diet. Their average weight was 130 g. In Exps. 3 and 4 the animals were also of mature age (135 days) and had also been fed (for the previous 90 days) upon a partially deficient diet. Their average weights were 100 and 90 g. respectively.

Exps. 5 and 6 were confined to young animals. In Exp. 5 they were 43 and in Exp. 6, 33 days old. Their average weights were 77 and 49 g. respec-

tively. The experimental technique was that usually adopted in this laboratory and has already been described in this journal [Stammers, 1921].

Exps. 1 and 2 were carried on for 101 days, Exps. 3 and 4 for 73 and Exps. 5 and 6 for 87 days.

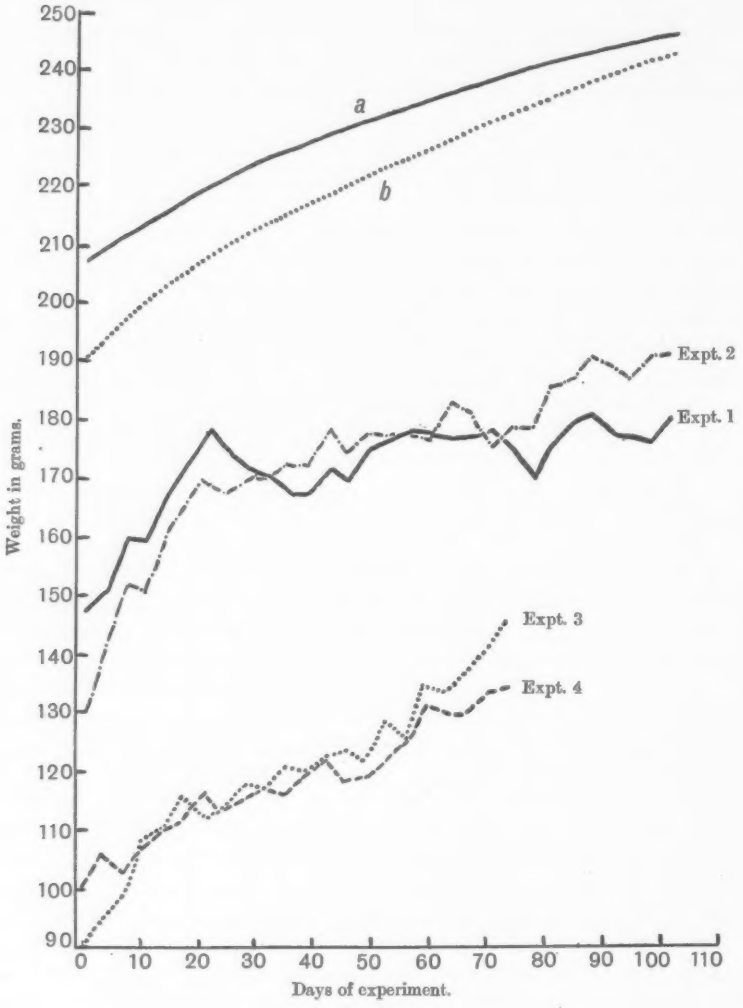

Fig. 2.

a, Normal for Expts. 1 and 2. *b*, Normal for Expts. 3 and 4.
Expt. 1 ——— Expt. 2 -·-·-· Expt. 3 ····· Expt. 4 – – – –

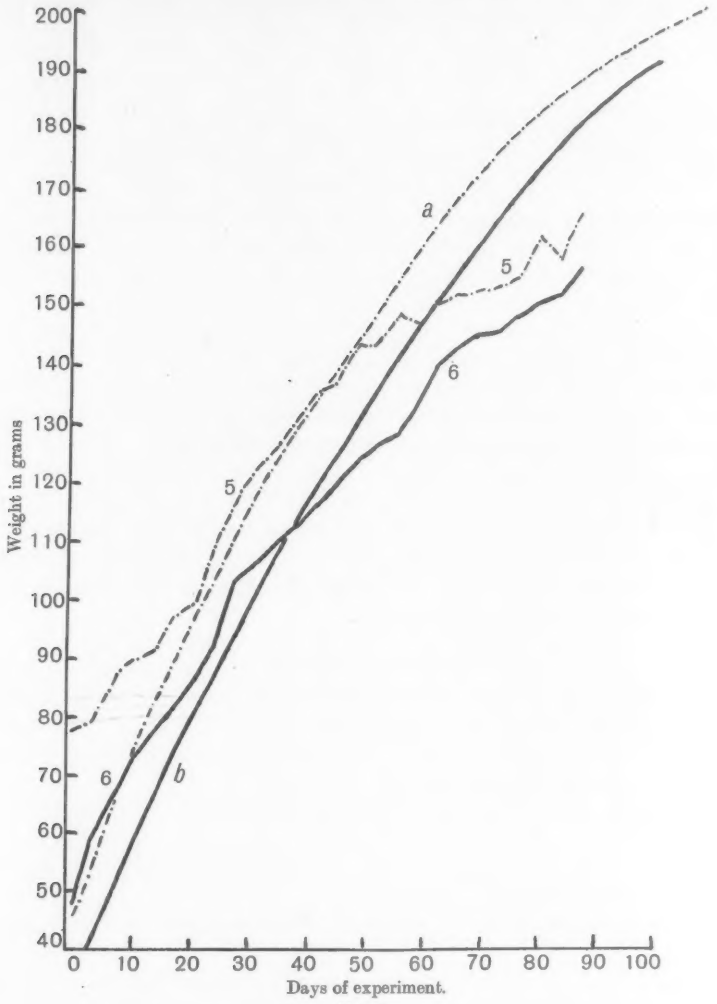

Fig. 3.

a, Normal for Exp. 5. *b*, Normal for Exp. 6.
-·-·-· Expt. 5 (cod-liver oil). ——— Expt. 6 (cod-liver oil).

Results.

The results are given in tabular form and graphs are also appended (Figs. 2 and 3) which show Donaldson's normal curves in addition to the growth recorded.

Exp.	Previous treatment	Age at commencement	Weight at commencement	Weight at conclusion	Actual increase	Normal increase (for age at commencement)
		days	g.	g.	g.	g.
1	Butter-fat	160	148	180	32	37
2	Partially deficient	160	130	191	61	37
3	Partially deficient	135	100	133	33	41
4	Partially deficient	135	91	145	54	41
5	None	43	77	164	87	141
6	None	33	48	156	108	144

Taking the first four experiments, *i.e.* those in which mature animals were used, it will be seen that in Exps. 1 and 3 the growth was subnormal, while in Exps. 2 and 4, although it was supernormal (for age) the growth shown was not nearly sufficient to cause the curves to approximate to that of normal growth—in other words, although cod-liver oil is probably the richest source of vitamin *A* available, its administration in these cases was not attended by any very marked or maintained increase in weight. The less refined sample, used in Exp. 3, which, *ceteris paribus*, would be expected to give the best results, actually proved the least effective.

As regards Exps. 5 and 6 in which young animals just weaned were used, the growth was slightly subnormal in each case although the animals were considerably above normal weight at the commencement of the experiment: the curves in each case fell off gradually and crossed the normal between the seventh and eighth weeks. When the experiments were terminated, however, they were still on the up grade.

Summary and Conclusions.

Two samples of cod-liver oil, one more highly refined than the other, were tested upon rats of mature age, some of which had previously been fed on a diet partially deficient in vitamin *A*. The more highly refined oil was also tested on young animals.

In the latter case (Exps. 5 and 6) the growth registered was *slightly* subnormal and, in view of the potency of cod-liver oil as a source of vitamin *A*, expectations were hardly realised. The sample used was, however, highly refined and this may be responsible.

As regards the first four experiments, No. 3 may be considered first. Although the sample used in this case was fairly crude, it failed to restore deficiency animals to normal weight even after a period of 73 days, moreover there was no sharp initial rise in the growth curve as might have been expected.

In the other experiments an initial rise did take place, but after three weeks the curves flattened out and the growth recorded ran roughly parallel to the normal (for rats of that age). Exp. 2 showed the best results, but even after 101 days the animals averaged 54 g. below the normal.

The explanation offered for these results is (*a*) that the growth impulses of the animals concerned in these experiments had, owing to their ages (135 and 160 days), been deprived to a great extent of their power to react to the

stimuli applied even by a fat rich in vitamin *A* and consequently they failed to show substantially greater growth than the normal for their age, or (*b*) that the oil had in the process of refinement lost a large part of its original vitamin *A*.

Point is given to the former theory by the fact that in Exp. 1 the animals had previously been fed on a diet containing butter-fat, which would, in itself, not be expected to impair the growth impulse.

I am indebted to Messrs Lever Brothers, Limited, for permission to publish this research.

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LXIX. ON THE CARDIAC, HAEMOLYTIC AND NERVOUS EFFECTS OF DIGITONIN.

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DIGITONIN, a sapo-glucoside found in *Digitalis purpurea*, has certain well-known properties—it causes haemolysis, forms a combination with cholesterol and some other sterols and has a marked action upon cardiac muscle.

The objects of the present investigations were (1) to compare the effects of certain allies of cholesterol upon the cardiac and haemolytic actions of digitonin and (2) to ascertain whether the activities of a digitonin solution are modified by shaking with an indifferent adsorbent.

The particular digitonin used was prepared and carefully purified by Mr J. A. Gardner, whom I desire to thank for his kind help.

In the first place it was necessary to measure both the cardiac and the haemolytic activity of the preparation.

Haemolysis. Graduated amounts of digitonin, in each case dissolved in 4 cc. of 0·95 % NaCl solution, were placed in a series of test tubes and 1 cc. of a 10 % dilution of defibrinated ox blood in physiological salt solution was added to each tube, so that all reactions took place in 5 cc. of fluid. The tubes were left standing in the laboratory for about 18 hours and then the condition of their contents was noted, *i.e.* whether haemolysed or intact. In Table I the results of such a test are set out. Repeated experiments showed that the minimum quantity of the digitonin which would, under the above conditions, completely lake 0·1 cc. of ox blood was from 0·0032–0·0036 mg., corresponding to a dilution of the digitonin of from 1 : 156,000 to 1 : 139,000. With cat's blood the minimum complete laking dose was rather less than 0·004 mg.

Table I. *Haemolytic power of Gardner's digitonin.*

Digitonin 0·004 %	NaCl 0·95 %	Ox blood 10 %	Dilution of digitonin	Amount of digitonin	Condition after 20 hrs. at room temp.
0·4 cc.	3·6 cc.	1 cc.	1 : 312,500	0·0016 mg.	Intact
0·5	3·5	1	1 : 250,000	0·0020	Very slight haemolysis
0·6	3·4	1	1 : 208,000	0·0024	Slight haemolysis
0·7	3·3	1	1 : 178,577	0·0028	Nearly haemolysed
0·8	3·2	1	1 : 156,250	0·0032	Very nearly haemolysed
0·9	3·1	1	1 : 139,000	0·0036	Completely haemolysed
1·0	3·0	1	1 : 125,000	0·004	„ „

Evaporation to dryness on a water-bath of a digitonin solution, whether in water or 90 % alcohol, did not affect the haemolytic power (Table II) but the presence of NaCl in hypertonic solution increased the resistance of the red cells to haemolysis by digitonin (Table III), as was previously observed by Luger [1921] for saponin.

Table II. *Digitonin solution evaporated to dryness and redissolved.*

Digitonin 0·004 %	NaCl 0·95 %	Ox blood 10 %	Condition after 18 hours
0·4 cc.	3·6 cc.	1 cc.	Trace of haemolysis
0·6	3·4	1	Partial ,,
0·8	3·2	1	Complete ,,
0·9	3·1	1	,, ,,

Table III. *Protective action of hypertonic* NaCl.

Digitonin	NaCl	10 % blood	Condition after 18 hours at room temp.
·004 mg.	4 cc. ·95 %	1 cc.	Complete haemolysis
·004	4 cc. 2·5 %	1	Partial ,,
·004	4 cc. 6·0 %	1	No ,,
·008	4 cc. 5·0 %	1	Complete ,,

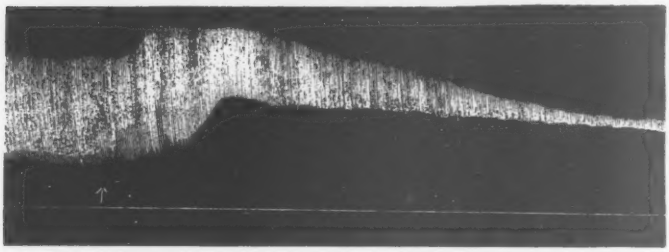

Fig. 1. Frog's heart perfused with Ringer's fluid. At arrow perfusion changed to Ringer + 0·004 % digitonin.

Cardiac action. Solutions of the digitonin in oxygenated Ringer's fluid were perfused through frogs' hearts *in situ* from a cannula inserted into the inferior cava, the fluid passing at a constant and moderate pressure through the heart and escaping by the cut aorta. A number of such perfusions showed that 0·0001 % of digitonin had very little or no effect; 0·001 % produced increase in the beat followed by rise of tone, diminution of diastole and finally stoppage or nearly so: 0·004 % produced the same effects somewhat more quickly (Fig. 1). Evaporation of a watery solution to dryness on a water-bath and redissolving in Ringer's fluid did not affect the activity. Perfusion of a frog's heart with a Ringer's fluid modified by omitting Ca quickly causes diminution of efficiency; if now, in the continued absence of Ca, 0·001 % digitonin is added to the perfusing fluid the normal efficiency is first partly restored and then the characteristic effect of digitonin is developed.

It is well known that digitonin treated with cholesterol loses its haemolytic power, it seemed therefore of interest to ascertain in how far bodies more or less closely allied to cholesterol such as phytosterol, β-cholestanol, coprosterol and pseudo-coprosterol affect the action of digitonin upon the blood and upon the heart.

Experiments with Substances allied to Cholesterol.

Method. A solution containing 0·004 % (or other percentage) of digitonin in 90 % alcohol was mixed with an equal quantity of a solution of the same strength of one of the cholesterol allies, also in 90 % alcohol. The mixture was evaporated to dryness on a water-bath and the dry residue taken up in so much Ringer's fluid as would give a possible concentration of digitonin and the sterol equal to that of the original alcoholic solution. This solution of the dry residue was filtered and the filtrate tested for its haemolytic power and cardiac effect. The results obtained with phytosterol, β-cholestanol, and coprosterol were identical—in each case both the haemolytic effect and the cardiac action were either cut out or greatly diminished (haemolytic effect, Table IV). On the other hand, the mixture digitonin + pseudo-coprosterol retained both its haemolytic and cardiac activity.

Table IV. *Effect of phytosterol, β-cholestanol, coprosterol and ψ-coprosterol.*

Possible concentration of digitonin 0·004 %.

	Solution of residue	NaCl 0·95 %	Ox blood 10 %	Dilution of digitonin	Condition after 18 hrs. at room temp.
Phytosterol or β-Cholestanol or Coprosterol	1 cc.	3 cc.	1 cc.	1 : 125,000	No haemolysis
	2	2	1	1 : 62,500	,,
	4	—	1	1 : 31,250	,,
ψ-Coprosterol	1	3	1	1 : 125,000	Complete haemolysis

Control for above: Digitonin 0·004 % in 90 % alcohol evaporated to dryness and taken up in NaCl, so as to give a possible 0·004 % digitonin.

Solution of residue	NaCl 0·95 %	Ox blood 10 %	Dilution of digitonin	Condition after 18 hrs. at room temp.
0·6 cc.	3·4 cc.	1 cc.	1 : 208,333	No haemolysis
0·8	3·2	1	1 : 156,250	Partial haemolysis
1·0	3	1	1 : 125,000	Complete ,,

When stronger solutions of digitonin were mixed with correspondingly increased amounts of phytosterol, β-cholestanol or coprosterol the protection against haemolysis and cardiac action was still complete (Table V).

Table V. *Protective action of phytosterol, β-cholestanol or coprosterol.*

Possible concentration of digitonin 0·1 %.

Solution of residue	NaCl 0·95 %	Ox blood 10 %	Dilution of digitonin	Condition after 18 hrs. at room temp.
0·5 cc.	3·5 cc.	1 cc.	1 : 10,000	No haemolysis
1·0	3·0	1	1 : 5,000	,,
4·0	—	1	1 : 1,250	,,

If the haemolytic test was carried out with mixtures containing a certainly haemolytic dose of digitonin and a gradually diminishing amount of phytosterol, β-cholestanol or coprosterol, the effect of these bodies in diminishing and finally extinguishing the haemolytic effect of the digitonin (Table VI) was perhaps still more strikingly in evidence.

Table VI. *Protective action of phytosterol.*

Digitonin 0·01 %		Phytosterol 0·01 %		Cat's blood 10 %	Condition after 20 hrs. at room temp.
0·5 cc.	+	0·5 cc.	Evaporated to dryness and 4 cc. NaCl added to each, then	1 cc.	No haemolysis
0·5	+	0·4		1	,,
0·5	+	0·3		1	,,
0·5	+	0·2		1	,,
0·5	+	0·1		1	Some haemolysis
0·5	+	0·05		1	Complete haemolysis
0·5		—		1	,,

In all the above experiments the solutions used were of equal percentage for the digitonin and the cholesterol allies; now the molecular weight of Gardner's digitonin is 1202, that of cholesterol and its isomer phytosterol is 386, so that when equal percentages by weight are used 1 phytosterol corresponds molecularly to 3·1 of digitonin. It was found that 0·31 cc. of a 0·01 % solution of digitonin (0·0031 mg.) will very nearly or sometimes completely haemolyse 0·1 cc. of ox blood. If the relationship between digitonin and cholesterol in the digitonin-cholesteride is monomolecular [Windaus, 1910], then 0·31 cc. of a 0·01 % solution of digitonin should be inactivated by 0·1 cc. of a 0·01 % solution of phytosterol or β-cholestanol or coprosterol (since the difference between the molecular weight of phytosterol, 386, and that of β-cholestanol or its isomer coprosterol, 388, is too small to be of importance in the conditions of the experiment); moreover multiples of these quantities should also be inactive. On the other hand, if the mixtures contain relatively an excess of digitonin more or less haemolysis should occur. In Table VII the results of an experiment carried out on these lines are shown. It will be seen that when molecular equivalents were mixed the neutralisation of the haemolytic action of the digitonin was complete, even when multiples of the minimum haemolysing dose were employed; when, however, the digitonin was relatively in excess haemolysis occurred and was most marked in the tubes containing the smallest relative proportion of the sterol.

As regards the cardiac activity it was found that the effect upon the heart ran parallel with that upon the red cells, the non-haemolytic mixtures had no cardiac effect and when the haemolytic action was not completely cut out there was also some cardiac effect. The results recorded in Table VII are strongly reminiscent of what occurs when diphtheria or other toxins are mixed with their respective antitoxins, and might perhaps be explained as simple adsorption phenomena (see below) were it not that other evidence points to a definite chemical union between digitonin and cholesterol. Moreover the fact that pseudo-coprosterol does not interfere with either the

haemolytic or the cardiac action of digitonin strongly supports the chemical view. In view of the chemical relationship between cholesterol and cholalic acid a series of experiments was made with this substance on the same lines as those recorded above, using a 0·01 % solution of the acid with 0·01 % of digitonin, but no evidence was obtained which would indicate that cholalic acid has any influence either upon the haemolytic or the cardiac action of digitonin.

Table VII.

All reactions in 5 cc. of fluid.

0·01 % sol. of coprosterol	0·01 % sol. of digitonin	Cat's blood 10 %		Condition after 18 hrs. at laboratory temp.
0·1 cc.	0·31 cc.	1 cc.	Molecular equivalents	No haemolysis
0·2	0·62	1		,,
0·4	1·24	1		,,
1·0	3·1	1		,,
0·05	0·31	1	Digitonin in excess	No haemolysis
0·1	0·62	1		Trace of haemolysis
0·2	1·24	1		Partial ,,
0·5	3·1	1		Complete ,,
0·2	3·1	1	Digitonin in excess, constant, coprosterol varied	Completely haemolysed
0·4	3·1	1		,, ,,
0·6	3·1	1		Nearly ,,
0·8	3·1	1		No haemolysis
—	0·1	1	Controls for digitonin	No haemolysis
—	0·2	1		Some ,,
—	0·3	1		Complete haemolysis
—	0·4	1		,, ,,
—	0·5	1		,, ,,

Adsorption Experiments.

The importance of adsorption in many pharmacological problems suggested experiments to ascertain if the solutions of digitonin are affected by indifferent adsorbents. To this end experiments were carried out with animal charcoal, kaolin and starch.

Method. 50 cc. of 0·004 % solution of digitonin in distilled water were gently shaken with 2 g. of purified animal charcoal (washed and dried) or kaolin or starch for 30 mins., then filtered and to the filtrate salts to make Ringer's fluid were added; this isotonic solution was then tested as to its haemolytic and cardiac activities.

Charcoal. The digitonin solution before treatment with charcoal, but with salts for Ringer's solution added, caused haemolysis as usual (0·8 cc. completely); after charcoal 5 cc. had no haemolytic effect. As to cardiac action the solution which had been treated with charcoal had no effect, but when the perfusion fluid was changed to the digitonin solution untreated with charcoal the usual digitonin effect appeared at once. The charcoal remainder was shaken with 25 cc. 90 % alcohol for 30 mins., the alcohol evaporated and the residue taken up in 50 cc. Ringer's fluid, this solution caused haemolysis and had a marked cardiac action. It is evident that animal charcoal very effectively adsorbs digitonin. If the period during which the charcoal

was applied or the amount of charcoal used was diminished the removal of the digitonin was not so complete.

Starch. In this case the digitonin solution was 0·001 %. This solution perfused through a frog's heart gave rise to increased systole and rise of tone, after treatment with starch the filtrate showed only a slight remnant of digitonin action. As to haemolysis, the results of the test are given in Table VIII and show that whereas before treatment with starch 1 cc. of the digitonin solution caused complete haemolysis, after starch 5 cc. did not do so. Digitonin then is adsorbed by starch and the filtrate from the starch is found to have lost both in haemolytic and cardiac activity.

Table VIII.

Before starch	1 cc.	of	0·001 %	digitonin	gave	complete haemolysis
After	,, 1	,,	,,	,,	,,	no haemolysis
,,	,, 2	,,	,,	,,	,,	,, ,,
,,	,, 3	,,	,,	,,	,,	trace only
,,	,, 5	,,	,,	,,	,,	nearly complete haemolysis

Kaolin. In this case also the digitonin solution used was 0·001 %. Before treatment with kaolin 1 cc. completely haemolysed 0·1 cc. of ox-blood, after kaolin 5 cc. caused only partial haemolysis (Table IX). As to cardiac action, before kaolin the solution gave a characteristic digitonin effect, after kaolin there was still some evidence of digitonin effect but much less marked. Clearly, then, kaolin adsorbs digitonin from watery solution.

Table IX.

Before kaolin	1 cc.	of	digitonin	sol.	gave	complete haemolysis
After	,, 2	,,	,,	,,	,,	no haemolysis
,,	,, 4	,,	,,	,,	,,	trace of haemolysis
,,	,, 5	,,	,,	,,	,,	incomplete haemolysis

At first glance it would appear as though of the three adsorbents animal charcoal were the most effective but much stress cannot be laid upon this point, since no attempt was made to insure the same number and size of particles, *i.e.* surface area, in the amounts of each of the adsorbents. Nevertheless there are good grounds for believing that charcoal occupies a peculiarly favourable position among adsorbents [Michaelis and Rona, 1920].

Windaus founded his method of estimating cholesterol by means of digitonin upon his discovery that cholesterol forms with digitonin a stable compound which is not haemolytic and dissolves with difficulty in alcohol and upon the fact that cholesterol esters [Hausmann, 1905] do not form such a compound. He considered the digitonin-cholesteride to be an addition product and not an ester and gave its formula as $C_{55}H_{94}O_{28}$ (digitonin) + $C_{27}H_{46}O$ (cholesterol), regarding it as a combination of one molecule of cholesterol with one of digitonin without loss of water. The results obtained in the present investigation are confirmatory of this view, since they show that when phytosterol, coprosterol or β-cholestanol are mixed in monomolecular proportions with digitonin the latter loses completely its haemolytic power,

whereas, if the mixtures contain less than this proportion of the sterol, more or less haemolysis takes place according to the degree of deficiency.

But now the very interesting question arises as to how the cardiac action of digitonin is affected. In the first place it may be noted that the effect of phytosterol, β-cholestanol or coprosterol upon the cardiac action runs strictly parallel to their effect upon the haemolytic power of digitonin—with monomolecular proportions both the haemolytic and the cardiac actions were cut out, whereas in mixtures containing a deficiency of sterol the more nearly the haemolytic action was neutralised the less was the cardiac effect and *vice versa*. Further, pseudo-coprosterol which does not prevent haemolysis, *i.e.* presumably does not combine with digitonin, did not affect the cardiac action either. These facts suggest that the action of digitonin upon the heart muscle depends upon the same factor as that which determines the haemolytic effect, viz. that there is present in cardiac muscle a body resembling or identical with cholesterol or coprosterol, etc., and that this sterol is at any rate in part free and not esterified. Moreover it has so important a function to perform in the muscle that its withdrawal as digitonin-sterol profoundly affects the physiological activity of the muscle cell. This supposition receives important confirmation from the fact that pseudo-coprosterol, which does not form a digitonide and does not affect haemolysis, has no effect upon the cardiac action of digitonin.

To show that the heart muscle probably does fix digitonin, the following experiment was carried out; a kitten was killed by pithing the brain and bleeding, the blood being collected and defibrinated. The heart was removed, washed free from blood, and the coronary vessels washed out with Ringer. The excised heart (7 g.) was pounded to as smooth a paste as possible, then well mixed with 50 cc. 0·002 % digitonin in Ringer and the mixture placed in the ice-chest. After 20 hours the mixture was centrifuged, the liquid poured off and divided into two parts, one of which was used to test haemolysis (Table X); with the other a frog's heart was perfused, with the result that both the haemolytic and the cardiac effects were markedly lessened.

Table X.

Heart emulsion + digitonin 0·002 %	Ringer	Kitten's blood 10 %	After 20 hrs.
0·8 cc.	4·0 cc.	0·2 cc.	Intact
1·0	3·8	0·2	,,
2·0	2·8	0·2	,,
5·0	—	0·2	,,
Digitonin sol. 0·002 %			
0·8 cc.	4·0 cc.	0·2 cc.	Partly haemolysed
1·0	3·8	0·2	Nearly completely
2·0	2·8	0·2	Completely

It may be recalled that many of the saponins, including digitonin, are powerful fish poisons, acting by paralysing the C.N.S., but that the cholesterol-saponide is non-toxic as well as non-haemolytic. In like manner saponin,

e.g. agrostemma saponin, found in the corn cockle, when it causes poisoning in human beings does not kill by its action upon the blood but by its effect upon the C.N.S. and upon the heart. On the other hand, tetanus toxin, besides its characteristic action upon the C.N.S., has definite haemolytic powers, indeed tetanus toxin is usually regarded as a mixture of tetanospasmin and tetanolysin. Cholesterol neutralises tetanolysin without diminishing the specific action of tetanospasmin. So the question arises; is the central nervous effect of saponin due to the same factor as enables it to cause haemolysis and alteration in cardiac muscle or do the conditions resemble those in tetanus toxin? To throw light upon this point the following experiment was carried out. A young rabbit was killed by a blow on the neck, the carotids were then cut and the blood collected and defibrinated; brain removed (6·7 g.), rubbed

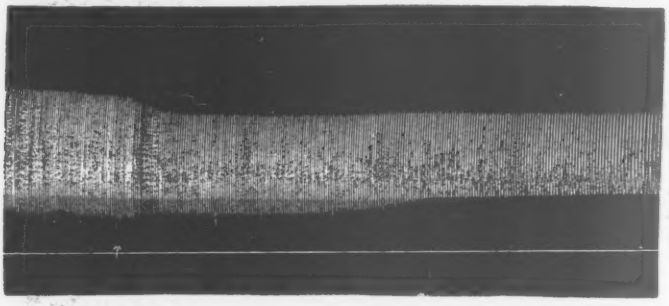

Fig. 2. Frog's heart perfused with Ringer's fluid. At arrow perfusion changed to emulsion of rabbit's brain in Ringer + 0·004 % digitonin. Compare with Fig. 1.

to a smooth paste in a mortar and then thoroughly mixed with 50 cc. of a 0·004 % solution of digitonin in Ringer's fluid; the emulsion was placed in the ice-chest for 20 hours; then well centrifuged, the supernatant fluid poured off and divided into two parts, one of which was used to perfuse a frog's heart, the other to test the haemolytic power. Table XI shows the results of the haemolytic test, the perfusion tracing is shown in Fig. 2. It will be seen at once that the digitonin-brain emulsion has no haemolytic power, at least not up to 5 cc. which is very much more than the ordinary haemolytic dose, and further the heart tracing shows merely a trace of digitonin action.

Table XI. *Protective action of brain substance.*

Possible concentration of digitonin, 0·004 %.

Brain + digitonin	Ringer	Ten rabbits' blood	After 18 hrs.
0·6 cc.	4·2 cc.	0·2 cc.	Intact
0·8	4·0	0·2	,,
1·0	3·8	0·2	,,
2·0	2·8	0·2	,,
5·0	—	0·2	,,
Digitonin 0·004 %			
0·8 cc.	4·0 cc.	0·2 cc.	Completely haemolysed

Considered in conjunction with the previous experimental evidence, these results appear to justify the conclusion that the action of saponin in causing narcosis in fishes and paralysis of the C.N.S. in man is an analogous phenomenon to digitonin haemolysis and digitonin cardiac action, *i.e.* there is free and unesterified in the brain a body allied to or identical with cholesterol, phytosterol, etc., which unites with digitonin. Moreover the function which this cholesterol-like body has to perform is of such importance that when it is inactivated by combination with digitonin (saponin) serious central nervous symptoms ensue.

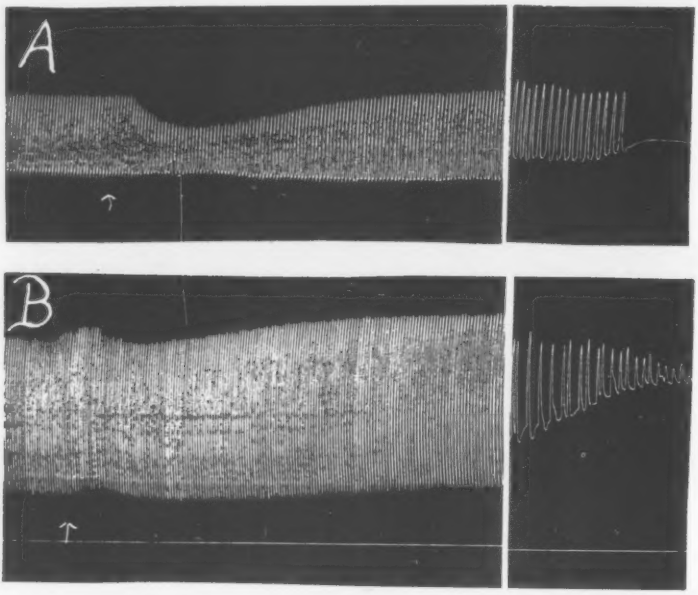

Fig. 3. Two frogs' hearts perfused with Ringer's fluid. *A*, at arrow perfusion fluid changed to emulsion of cat's brain in Ringer + 0·001 % strophanthin. *B*, at arrow perfusion fluid changed to Ringer + 0·001 % strophanthin. In each case the break in the tracing represents an interval of 15 mins.

Kobert [1904] has shown that the toxic action of saponin as well as the cardiac effects is abolished by the combination of saponin with cholesterol, and further that the extreme toxicity of sapotoxin solutions for fishes is entirely lost in the cholesterol-sapotoxin combination.

The effect of digitonin is also neutralised by adding milk to the digitonin solution.

It is interesting to note that strophanthin, which is a glucoside but not a saponin, does not lose its cardiac action when brain emulsion is added to the strophanthin solution (Fig. 3).

SUMMARY.

Monomolecular combinations of digitonin with phytosterol, coprosterol, β-cholestanol are non-toxic for red blood cells and for the frog's heart; any excess of digitonin beyond this proportion can be detected by the action of the solution upon red cells and upon the heart. Pseudo-coprosterol does not effect the activity of digitonin. Digitonin mixed with an emulsion of brain substance loses its toxicity for red cells and for the heart.

It is suggested that the toxic action of digitonin upon red blood cells, cardiac muscle cells and cells of the central nervous system (fishes) is essentially the same—it attacks a substance allied to or identical with cholesterol which is present free and not esterified in these various cells. This free sterol has in each case so important a function to perform that its inactivation by combination with digitonin leads to a profound modification of the physiological activities of the respective cells.

Digitonin is readily adsorbed from solution in water (*e.g.* 1 : 25,000) by animal charcoal, kaolin or starch; the adsorption by charcoal appears to be particularly complete.

Cholalic acid has no effect upon the action of digitonin.

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LXX. THE HEAT-COAGULATION OF PROTEINS.

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It has been long pointed out that the heat-coagulation of proteins is not so simple a phenomenon as it at first may seem to be. Schmidt and his pupils, Aronstein [1874], Heynsius [1874], Kieseritzky [1882] and Rosenberg [1883], showed that albumin solutions from which the electrolytes had been removed by dialysis did not coagulate on heating; heat-coagulation could only be produced after the addition of electrolytes to such solutions; it took place also if a small amount of any salt was added to the cold albumin solutions which had been preliminarily heated. These authors therefore suggested the hypothesis that heating may transform the albumin into an unknown body, the solutions of which coagulate on the addition of a very small quantity of salts [Rosenberg, 1883, p. 34].

Hardy [1899, 1900, 1906] showed later that in solutions of protein altered by heating ultramicrons appear and that the protein can now be precipitated by adjusting the reaction so as to render the particles isoelectric with the solution. When the protein is made negative by adding alkali the valency of cations is of importance for the precipitation; on the contrary, the cations have no part in the precipitation if protein is made positive.

Then Michaelis came to the conclusion [Michaelis] that the properties of albumin solutions may be changed by heating from those of an "emulsoid" to those of a "suspensoid," whereas the high viscosity and the low surface-tension of solutions of albumin altered by heating point rather to their hydrophilic character. These solutions probably do not fall into either category of the colloidal state but form an intermediate group with some of the properties of both.

Chick and Martin [1913, 1] who investigated the heat-coagulation of proteins and the influence of various factors upon its course confirm the conclusion that this phenomenon consists of two distinct processes: (1) "denaturation" and (2) "agglutination" of the denaturated protein by electrolytes. They regard the former as a reaction between protein and hot water without determining this reaction more precisely.

Contradicting the results of the above investigations and of his own earlier experiments Pauli [1899, 1908] affirms that he has observed that protein solutions free from electrolytes ("amphoteres Eiweiss") coagulate on heating and that the addition of electrolytes to such solutions increases their coagulation temperature. Criticism and detailed consideration of this new opinion may be postponed till some experiments related to heat-coagulation have been described.

A number of scattered observations indicated that temperature is an accelerating factor in the process of heat-coagulation. Buglia [1909] was the first who precisely determined the dependence of the velocity of heat-coagulation upon the temperature, and found this dependence to be logarithmic.

Chick and Martin [1910] found that the coagulation-rate increases 1·3–1·9 fold per 1° temperature-rise. Such a high temperature coefficient bears only on the denaturation, while the agglutination-velocity does not increase so rapidly with the temperature as the denaturation-rate, its temperature coefficient being equal to 2·5 per temperature-rise of 10° [Chick and Martin, 1913, 2]. These authors showed moreover that, if precautions are taken to keep the concentration of hydroxyl or hydrogen-ions constant during the process, the denaturation may proceed as a reaction of the first order.

(1) Experimental Methods.

My experiments were made before I was acquainted with the papers by Chick and Martin, and the method used by me differs from theirs. I find however that it is more convenient for observing slight, and sometimes short, alterations of velocity in both the processes that are comprised in heat-coagulation.

In order to estimate alterations in the velocity of heat-denaturation and in coagulation ("agglutination") of the denaturated protein under influence of various factors, in my experiments, the time required to produce a slight turbidity in the protein solutions like that of a ground glass (chosen for all the experiments) was noted. The solution to be tested was usually introduced by means of a capillary pipette into a small vessel made of two cover-glasses ($20 \times 10 \times 0{\cdot}1$ mm.) cemented with oil-lacquer (linseed-oil and minium) on both sides of a rectangular brass plate (0·7 mm. thick, 22 mm. long, 13 mm. broad) the middle of which was rectangularly cut out, so that the rim-breadth (at three sides) was equal to 3 mm.

A drawing of this vessel is given in one of my previous papers devoted to the reactions between starch and water brought about by heating[1]. There are also given a drawing and a photograph of the thermostat used.

[1] Lepeschkin, "Etude sur les réactions chimiques pendant le gonflement de l'amidon dans l'eau chaude." Le manuscrit est renvoyé à M. le professeur Chodat (Génève, Suisse) pour la publication dans l'*Archives des sciences naturelles.*

The above vessel with the protein solution to be tested was immersed in the water of the thermostat, in which the temperature variation did not surpass 0·03°. Close by the vessel, a similar rectangular brass plate (cut out in the middle) was placed; instead of the cover-glasses (see above) a piece of ground glass of the same size was cemented on one side of this plate. Both (viz. the vessel and the plate with ground glass) were observed through the front glass-wall of the thermostat box by means of a horizontal microscope of which the eye-piece was removed and the objective was substituted by a black plate having two rectangular apertures corresponding respectively to the front cover-glass of the vessel and to the ground glass. The protein solution and the ground glass were illuminated by an electric lamp (50 candles) through the back glass-wall of the thermostat, and the microscope-tube was situated obliquely to the front glass-wall (in order to observe most easily the turbidity produced in the protein solution by heating).

The water of the thermostat was uninterruptedly stirred by means of an electric stirrer.

The time-intervals were defined by means of a seconds pendulum.

The preliminary experiments showed that the liquid contained in the above vessel takes up the temperature of the thermostat water in about 20 seconds. This interval of time was always deducted from the time required to produce the above turbidity of the solution to be tested.

The material used was filtered egg-white, egg-albumin Kahlbaum from which electrolytes had been removed by dialysis, serum-albumin Kahlbaum similarly treated or purest recrystallised egg-albumin.

The first experiments are concerned with denaturation-velocity at various temperatures.

(2) The temperature coefficient of denaturation.

Preliminary experiments showed that the coagulation-velocity of the denaturated proteins in solutions containing a sufficiently great quantity of electrolytes is extraordinarily great in comparison with the denaturation-velocity at the same temperature. It could thus be assumed that the coagulation of denaturated protein proceeds instantaneously at the temperatures the influence of which upon the denaturation was investigated in my experiments. The appearance of the turbidity in the protein solutions containing a sufficient quantity of electrolytes marked, therefore, the denaturation produced by heating (or is it truer to say the degree of denaturation corresponding with the turbidity of the solution like that of the ground glass). The results were the following.

Filtered egg-white. The temperature of water in the thermostat was in the experiments: 63·01°, 62·01°, 61·02°, 60·03°, 59·03°, 58·01°, 57·04° and 56·00°. The average (from six experiments) times (in seconds) passed from the moment of immersing the vessel with egg-white into the water to the appearing of the required turbidity of the solution T were found respectively:

40 secs., 97 s., 230 s., 595 s., 1535 s., 3720 s., 9520 s., and 22,600 s. The temperature coefficients per rise of 1° thus were found to be: 2·4, 2·4, 2·5, 2·6, 2·4, 2·6, 2·4 or on the average 2·5.

3 % *solution of egg-albumin Kahlbaum.* This had been dialysed during 5 days and no longer coagulated on heating; in order to make it coagulable 0·4 % ammonium sulphate was added. Temperatures of the thermostat: 70·04°, 69·00°, 68·00°, 67·04°, 66·04°, 65·06°. The average (from five experiments) times T: 91 secs., 136 s., 207 s., 306 s., 452 s., 690 s. The average temperature coefficient per rise of 1° = 1·5.

2·5 % *solution of serum-albumin Kahlbaum.* This had been dialysed during 20 hours and no longer coagulated on heating. 0·7 % potassium chlorate was added. The temperatures were: 75·00°, 72·00°, 70·00°, 68·00°. The average times T: 59 secs., 205 s., 470 s., 1298 s. The average temperature coefficient = 1·6.

1 % *solution of recrystallised egg-albumin* (not coagulating on heating). 1·5 % ammonium sulphate was added. The temperatures were: 70·00°, 69·00°, 68·00°, 67·00°. The average times T: 154 secs., 331 s., 784 s., 1860 s. The average temperature coefficient = 2·3.

The coefficient of denaturation thus is equal to 1·5–2·5 *per* 1°, *viz.* 58–9540 *per* 10°. A similar coefficient was found by me for the chemical reaction between starch and water during the swelling of starch in hot water. See footnote p. 679.

(3) On the nature of denaturation. The influence of hydrogen- and hydroxyl-ions upon the denaturation-rate.

Chick and Martin's researches [1910] show that water is of great significance in the denaturation process of proteins, and reasoning from analogy with the above mentioned reaction between starch and water, I should incline, in accordance with Chick and Martin, to regard this process as a chemical reaction between protein and water. Further we shall see indeed, that the chemical properties of denaturated protein differ considerably from those of native protein.

In my cited paper it was suggested that during the swelling of starch in hot water no hydrolysis takes place but the starch forms a compound with water. Indeed, the velocity of the reaction between starch and water increases not at all proportionally to the hydrogen-ion concentration but depends upon the nature of the acid added, *e.g.* sulphuric acid, in weak concentrations, even lowers the reaction-rate. So then the hypothesis, that starch preliminarily forms a compound with acid before it reacts with water, must be adopted. The preliminary formation of compounds with potassium and sodium hydroxide which react more easily with water than free starch is also certain.

The fact that denaturation proceeds as a reaction of the first order (monomolecular reaction) indicates that the protein reacts with the solvent, but leaves it uncertain whether the denaturation is a hydrolysis, like the inversion

of sugar, or is accompanied by the formation of a compound between protein and water. It is important in this connection to know the effect of acids and alkalies upon the denaturation-velocity.

Chick and Martin [1913] who found the denaturation-rate to be increased with increasing concentration of hydrogen- or hydroxyl-ions in protein solutions suggest that the cause of this phenomenon lies in the fact that protein forms salts with acids and alkalies, and that these salts are in a more intimate association with water.

The researches of Hoffmann [1889, 1890], however, had already shown that slight quantities of acid which are added to a pure protein solution form no salts with protein, and this result is in accordance with the recently found weakly acid nature of proteins (about 0·00001 *N* hydrogen-ion concentration). Nevertheless my experiments showed that a very remarkable increase of denaturation-rate is observed even when albumin solutions contain 0·00016 mol. nitric acid per litre. The increasing effect of such small concentrations of acid upon the denaturation-rate cannot be explained except by an acceleratory influence of hydrogen-ions like that observed in the case of hydrolysis of carbohydrates, viz. by their catalytic properties. The denaturation-velocity was however found in my experiments not to be proportional to the concentration of hydrogen-ions present in the solution when strongly different concentrations of the same acid had been tested. It increased more slowly than the hydrogen-ion concentration when the latter was not greater than 0·002 mol. per litre, but it began to increase more rapidly when the hydrogen-ion concentration attained 0·01 mol. per litre.

However, if various acids were tested at about the same molecular concentrations, the denaturation-velocity was found to depend upon the hydrogen-ion concentration, so that contrary to the case of the reaction between starch and water, the nature of the acid has here no influence upon the denaturation-velocity.

The material used in my experiments was a solution of egg-albumin Kahlbaum which had been dialysed during many days. The solution was diluted partly with water, partly with nitric acid so that the albumin-content in all solutions was 0·66 %. In order to make the solutions obtained coagulable 0·8 % $(NH_4)_2SO_4$ was added. When the concentration of nitric acid was 0 *N*, 0·00016 *N* mol., 0·00032 *N*, 0·002 *N*, 0·016 *N*, the average time of coagulation at 63° was found respectively to be 2440 secs., 1550 s., 1220 s., 315 s., 11 s. In the case of different acids the average coagulation-time at 53° was found to be equal to 167 secs. in the presence of 0·016 *N* HNO_3 (viz. hydrogen-ion concentration = 0·015 *N*), 161 secs. in the presence of 0·015 *N* HCl (hydrogen-ion concentration = 0·014 *N*), 86 secs. in the presence of 0·018 *N* H_2SO_4 (hydrogen-ion concentration = 0·024 *N*) and 1275 secs. in the presence of 0·016 *N* acetic acid (hydrogen-ion concentration = 0·0016 *N*).

The absence of proportionality between the denaturation-velocity and the concentration of hydrogen-ions in the case of strongly different concen-

trations of the same acid, is not unexpected. Indeed a strict proportionality between the hydrogen-ion concentration and the reaction-rate also does not exist in the case of the hydrolysis of sugar by acids, particularly when salts are present in the sugar solution.

Arrhenius [1889] who investigated the influence of salts upon the inversion-velocity showed that the latter departs from its normal value almost proportionally to the salt-content of the solution; moreover, the weaker the acid concentration, the more considerable is the influence of salt; so for instance, the inversion-rate increased by 35–40 % on adding KBr to the concentration of 0·4 *N*, when hydrobromic acid was 0·002 *N*, and it increased by 200–240 % when the acid was 0·0005 *N*.

At the same time, it was necessary, in my experiments, to add ammonium sulphate to the albumin solutions to bring about the coagulation of denaturated albumin. No doubt the salt increased the catalytic power of the hydrogen-ions normally contained in the albumin solution, the presence of which is due to the electrolytic dissociation of the protein molecules (hydrogen-ion concentration of about 0·00001 *N*); then, the salt induced thereby an extraordinarily strong increase of the denaturation-rate[1]. However, this increasing effect of salt upon the catalytic power of hydrogen-ions strongly diminished on the further increase of the concentration of these ions by adding nitric acid so that the denaturation-rate increased only by 1·6 fold, while the hydrogen-ion concentration increased by 15 fold. By virtue of the same cause the denaturation-velocity continued to increase more slowly than the concentration of hydrogen-ions on the addition of acid. When, however, this concentration became more than 0·002 *N* the denaturation-rate began to increase more rapidly than the hydrogen-ion concentration (an increase of the latter of 8·3 fold was accompanied by an increase of the former of 28 fold). The same was found by Chick and Martin [1912].

The more rapid increase of the denaturation-rate by stronger concentrations of acid could not be explained except by the formation of compounds between albumin and acid which react more easily with water than the unaltered albumin, the acid concentration 0·016 *N* being evidently sufficient for the formation of such compounds.

As the increasing effect of salts upon the catalytic power of hydrogen-ions varies only with a very considerable alteration of concentration of these ions (Arrhenius, see above), it is very comprehensible that the denaturation-rate remained in my experiments almost proportional to the hydrogen-ion concentration in the case where denaturation proceeded in solutions containing similar concentrations of various acids.

In view of the importance of hydrogen-ions in the reaction between protein and water, it seems to be very probable that the denaturation is a slight hydrolysis of protein; such hydrolysis cannot be discovered by chemical

[1] It is uncertain whether this increase of denaturation-rate is due to the formation of an active albumin, analogically to the active sugar of Arrhenius.

analysis, the protein molecule being too large, and it is not surprising that Chick and Martin could obtain no evidence of hydrolysis by denaturation of pure proteins in the chemical way.

We go on now to consider the influence of alkali upon denaturation.

From Chick and Martin's experiments [1913] it follows that in the presence of very weak alkali concentration (hydroxyl-ion concentration equal to $10^{-3\cdot8}$ N) the denaturation proceeds too slowly and only on addition of more alkali (hydroxyl-ion concentration $10^{-2\cdot7}$ N) does the denaturation-rate increase so as to be comparable with that in the original, slightly acid solution. These experiments confirm therefore the above hypothesis that the denaturation of albumin, free from electrolytes, is accelerated by its own hydrogen-ions present in the solution in concentration of about 10^{-5} N: after the neutralisation of albumin by a small amount of alkali the accelerating effect of these ions is abrogated and the denaturation-rate strongly diminished. The strong increase of the latter on the addition of more alkali could then be explained by the formation of albumin salts with alkali (alkali albuminate) which react more easily with water than the unaltered albumin.

Unfortunately it was only possible for Chick and Martin to institute a comparison over a small portion of the curves expressing the dependence of the denaturation-rate upon the hydroxyl-ion concentration (namely, from a concentration equal to $10^{-3\cdot8}$ to $10^{-2\cdot7}$). Nevertheless it seemed to me to be desirable to investigate the effect of stronger concentrations of alkali upon the denaturation-velocity. My experiments made for this purpose showed that an increase of the hydroxyl-ion concentration beginning from $10^{-2\cdot2}$ N only influences the denaturation-rate very little. Thus an increase of alkalinity of over sixty-fold was accompanied by a decrease in denaturation-rate of 27 %.

In my experiments a solution of serum-albumin Kahlbaum dialysed during 6 weeks and containing 0·49 % albumin was used. This solution was diluted with an equal volume of a solution of potassium hydroxide of known concentration and in order to keep it coagulable ammonium sulphate (concentration of 0·2 N, or 0·4 N when the alkali concentration was great) was added to it. When the hydroxyl-ion concentrations were 0·006 N ($=10^{-2\cdot2}$), 0·012 N, 0·067 N, 0·150 N, 0·400 N, the average denaturation-times at 70° were found to be equal respectively to 270 secs., 285 s., 300 s., 320 s., 370 s.

The study of the influence of alkalies and acids upon the denaturation showed therefore that the above hypothesis concerning the nature of this reaction is true: the denaturation is a weak hydrolysis of protein.

This hydrolysis can be accompanied also by the splitting off of any amino-acids containing sulphur from the protein molecule [Moll, 1904] and by an increase in the amount of carboxyl group, for, as will be shown below, the acid properties of albumin increase after the denaturation.

Concerning the known increase of alkalinity of a solution of albumin,

after denaturation, Chick and Martin showed that the diminution of acidity during heat-coagulation takes place only in acid solutions, while in alkaline solutions a diminution of alkalinity is observed [Chick and Martin, 1913, 1]. It has already been pointed out that protein can unite with acids and alkalies before denaturation; the coagulation following the latter would then evidently remove not only protein from the solution but also a quantity of acid or alkali so that the acidity or the alkalinity of solution would be diminished during the heat-coagulation.

(4) Influence of salts upon the denaturation-velocity of protein.

As mentioned above, salts increase the catalytic power of hydrogen-ions and produce thereby an increase of inversion-rate of sugar. Salts probably also accelerate the denaturation of protein for they increase the catalytic power of hydrogen-ions whose presence in the electrolyte-free protein solution is due to the electrolytic dissociation of the protein molecules which are more acid than alkaline (see above, hydrogen-ion concentration of about $10^{-5}\,N$). Moreover, Arrhenius found the accelerating effect of salts upon inversion to increase with the increasing salt concentration. On the other hand, as is known, salts diminish the electrolytic dissociation of electrolytes, and they may thus diminish that of protein; moreover the researches of Lillie [1907] showed that salts considerably diminish the osmotic pressure of albumin solutions; that is to say they lower their degree of dispersion and, therefore, the particle-surface of the protein; so that the reaction between protein and water may be retarded.

We should therefore expect that an increase of salt concentration would accelerate the denaturation of albumin when the salt concentration is small, it would however diminish it when the salt concentration is great and it would leave it unaltered if the salt concentration is intermediate.

My experiments showed that this suggestion is right. But, unfortunately, they could not render clear the effect of very small concentration of salts upon the denaturation-velocity, the method employed having required the presence in the protein solution of an amount of salt sufficiently great to produce the coagulation of the denaturated albumin (see above).

The material used in my experiments was egg-albumin Kahlbaum, which had been dialysed many days (content 0·66 %). When the concentration of added ammonium sulphate was $0{\cdot}03\,N$, $0{\cdot}06\,N$, $0{\cdot}12\,N$, $1{\cdot}1\,N$ and $1{\cdot}32\,N$, the average times of denaturation at 70° were found respectively to be 932 secs., 185 s., 185 s., 270 s. and 605 s. These results are in accordance with the results of Chick and Martin, who investigated only the concentrations from 0·1 to 1 N and above.

As is known, salts accelerate the inversion of sugar by acid in different degree, according to their lyotropic properties, which are chiefly due to the effect of anions: $SO_4 < Cl < Br < SCN$ [Spohr, 1888]. It was therefore to be

expected that salts would accelerate the denaturation of acid solutions of protein in the same series of anions. Indeed, my experiments showed that the denaturation-velocity of protein in acid solutions is incomparably more increased by potassium thiocyanate than by potassium sulphate; potassium chlorate takes the middle position but it is, in its effect, nearer to potassium sulphate.

The material used was egg-albumin Kahlbaum, the 2 % solution of which had been dialysed during 5 days. The solution obtained was diluted with water and 0·1 *N* HNO_3, so that the content of albumin in all the solutions was 0·41 %, and the content of acid 0·01 mol. per litre. To the solutions such quantity of various salts was added as to render the salt-content of each solution equal to 0·083 mol. per litre. In the presence of KCNS, KCl, K_2SO_4 the average time of denaturation at 50° was found respectively to be 15 secs., 3000 s. and 4650 s.

If albumin solutions contain no free hydrogen-ions but are slightly alkaline, the presence of salts could not evidently accelerate the denaturation (there are no hydrogen-ions in the solution the hydrolytic power of which could be increased), and it is to be expected that the nature of the anion would have no influence upon the denaturation-velocity.

Indeed, my experiments showed that the denaturation-rate in this case is almost equal in the solutions containing salts of various anions. A slightly alkaline solution of egg-albumin Kahlbaum had been dialysed until it no longer coagulated on heating. When 0·1 *N* K_2SO_4, KCl, KI and $KOCOCH_3$ were added, it coagulated at 75° respectively in 212, 220, 225 and 227 secs.

Concerning the increasing influence of various cations upon the catalytic power of hydrogen-ions in the case of denaturation, it is almost the same for various alkali metals The acceleration of denaturation by salts of alkaline earths and heavy metals is however so strong, as to make necessary the suggestion that the salts in question first enter into chemical unions with protein which are soluble in water and react with it more easily than the free protein, and therefore are more easily denaturated. The other suggestion that alkaline earths and heavy metals form insoluble compounds with native protein (which are therefore no longer capable of denaturation by heating), the formation of which is accelerated by high temperature as are all other chemical reactions, is less probable, for the temperature coefficients of the reaction were found in my experiments to be too high: 1·28–1·38 per rise of 1°.

A solution of egg-albumin Kahlbaum, after dialysis during 6 days, was diluted with water and salt solutions so that all the solutions to be tested contained 0·3 % albumin. In the presence of 0·1 *N* KCl the average time of denaturation was found, at 75°, to be 2095 secs., while in the presence of 0·1 *N* $BaCl_2$ denaturation proceeded instantaneously at the same temperature; in the presence of 0·003 *N* $BaCl_2$ it required 95 secs., 0·003 *N* $MgCl_2$ 120 secs., 0·0001 *N* HCl 52 secs., while it proceeded instantaneously in the presence of 0·003 *N* HCl.

The study of the influence of salts upon denaturation showed, therefore, that there was no contradiction with the foregoing hypothesis.

(5) The cause and the temperature coefficient of the coagulation of denaturated protein.

As mentioned above, it has long been known, that protein solutions made heat-incoagulable by dialysis acquire the property to coagulate on heating when some electrolyte is added to them. They coagulate also on the addition of electrolytes when preliminarily heated and cooled. The presence of electrolytes is therefore required to produce the second process comprised in heat-coagulation. In order to render this process clear it was first of all necessary to answer the questions, whether the coagulation of denaturated protein is a pure physical process or, at the same time, also a chemical reaction; whether this process is, in its nature, like the other processes of coagulation of suspension-colloids by electrolytes.

In this connection it was interesting to estimate the temperature coefficient of coagulation of denaturated protein and to compare it with that of chemical reactions and of other coagulations.

My experiments showed firstly that the coagulation of denaturated protein proceeds at a certain temperature considerably more rapidly than the denaturation at the same temperature if the protein solution contains a sufficiently great amount of salt.

Thus, in one of my experiments, the average time required to produce the necessary turbidity of 0·57 % solution of dialysed egg-albumin Kahlbaum to which ammonium sulphate was added to the concentration of 0·05 *N* at 65° was found to be 2220 secs., while the average time required to produce the same turbidity of the same albumin solution which had been boiled and to which after cooling the same amount of ammonium sulphate was added (*i.e.* the time to produce the coagulation) was found to be 10 secs. at 65°.

Further, in accordance with Chick and Martin, my experiments showed that the average temperature coefficient of coagulation of denaturated albumin is usually equal to 1·1 (it varies from 1·08 to 1·2) per temperature-rise of 1°. But sometimes it increases to 1·3. The results of four of my experimental series are the following:

I. Solution of egg-albumin Kahlbaum made non-coagulable on heating (by dialysis) and containing 0·57 % protein was boiled. After cooling $(NH_4)_2SO_4$ was added to it to the concentration of 0·025 *N*. The average times of coagulation at 80°, 75°, 70°, 65°, and 60° were found to be equal respectively to 165 secs., 405 s., 943 s., 1922 s., 2800 s. The temperature coefficient = 1·15.

II. Solution of egg-albumin Kahlbaum. Content of albumin = 0·41 %. Content of ammonium sulphate added after boiling and cooling the solution = 0·05 *N*. The average times of coagulation: at 65° 10 secs., at 60° 40 secs., at 55° 138 secs., at 53° 251 secs., at 50° 687 secs., at 47° 1630 secs. The temperature coefficient = 1·33.

III. 0·14 % *solution of recrystallised egg-albumin*, made non-coagulable on heating by dialysis. Content of ammonium sulphate added after boiling and cooling to the solution = 0·1 *N*. The average times of coagulation were: at 45° 72 secs., at 40° 152 secs., at 35° 425 secs., at 30° 1200 secs. The average temperature coefficient = 1·2.

IV. Egg-white had been dialysed till the absence of heat-coagulation, diluted with water fivefold, boiled and cooled. $(NH_4)_2SO_4$ was then added to the concentration of 0·1 *N*. The average times of coagulation were: at 60° 35 secs., at 50° 297 secs., at 45° 687 secs., at 40° 1325 secs. The average temperature coefficient = 1·18.

As known, the denaturated protein is regarded as a suspension-colloid (see above). It was therefore interesting to compare the temperature coefficients found with those of coagulation of other suspension-colloids. But there are yet no investigations dealing with the dependence of coagulation upon temperature. Scattered up and down a number of observations only pointed out that colloidal solutions are generally less stable at higher temperatures [see, for example, Zsigmondy, 1912].

I therefore made some experiments with colloidal solutions of arsenic trisulphide and lecithin by means of the method above described, which, in the case of arsenic, was a little modified; the vessel for the colloidal solution was observed with the intact horizontal microscope (small magnification) and the time-intervals were noted which were required to produce a complete coagulation. This was marked by the appearance of orange particles of arsenic trisulphide, all through the liquid, on the pure white ground.

A 0·5 % colloidal solution of arsenic trisulphide (obtained by passing sulphuretted hydrogen into water containing powdered As_2O_3), from which H_2S had been removed by a current of air, was centrifuged until particles visible under the microscope were absent. Then ammonium sulphate was added to the resulting colloidal solution to the concentration of 0·01 mol. per litre.

The average times of coagulation were found to be at 85° 228 secs., at 75° 401 secs., at 65° 601 secs., at 55° 1110 secs. The temperature coefficient = 1·05 (per rise of 1°).

In another experimental series where 0·4 % solution of arsenic trisulphide was used and the concentration of added salt (NaCl) was 0·04 mol. per litre, the average times of coagulation were: at 60° 19 mins., at 50° 29 mins., at 40° 39 mins., at 30° 56 mins. and at 17·3° 86 mins. The temperature coefficient was therefore 1·04 (per rise of 1°).

1 % solution of lecithin in water was made by mixing an ethereal solution of lecithin with water and removing ether by blowing air through the solution. Twenty-four hours after the preparation ammonium sulphate was added to the solution (concentration 0·08 *N*). The average times of coagulation were at 90° 106 secs., at 80° 500 secs., at 70° 910 secs., at 60° 1960 secs. The average temperature coefficient was therefore 1·10. When the concentration of

lecithin was 0·5 %, the times of coagulation were: at 90° 376 secs., at 80° 606 secs., at 70° 1356 secs., at 60° 3290 secs. The average temperature coefficient was therefore 1·07 (per rise of 1°).

From the experiments cited it may be seen that the temperature coefficient of coagulation of denaturated albumin is greater than that of arsenic trisulphide or lecithin but it is nearer to that of lecithin, which is known to occupy an intermediate position between hydrophilic and hydrophobic colloids (resp. emulsoids and suspensoids). In the case of arsenic trisulphide the temperature coefficient of coagulation is like that of the diffusion of salts. The temperature-rise only increases the quantity of salt acting upon the colloidal particles. The temperature effect, in the case of lecithin coagulation, is however more complex. The temperature influences in this case not only the salt quantity acting upon the colloidal particles, but also the degree of dispersion and therefore the reacting surface of the colloidal particles, and the coefficient is greater.

The most complex effect of temperature is that upon the coagulation of denaturated protein. The temperature influences probably, in this case, not only the dispersion of the colloid and the acting salt quantity, but also the rate of reaction between salt and protein. For, a formation of loose chemical compounds ("adsorption compounds") of salts with denaturated protein during the coagulation is very probable (see below).

(6) Influence of acids and alkalies upon the coagulation of denaturated protein.

Denaturated proteins are known to be soluble in alkalies and in strong acids [Cohnheim, 1904], so that the formation of compounds of denaturated proteins with alkalies and acids is undoubted. From the solution of denaturated albumin in weak potassium hydroxide the protein can be precipitated by ammonium sulphate. The precipitate is however easily soluble in a stronger solution of this alkali, but a greater quantity of salt precipitates protein anew. This precipitate is also soluble in more alkali, etc. The increase of alkali concentration produces therefore a continuous change of colloidal properties of denaturated albumin owing evidently to the formation of alkali compounds of protein, and the greater the alkali concentration of the solution the more alkali these compounds contain.

We should therefore expect that alkalies and acids would have a considerable influence upon the coagulation-velocity of denaturated albumin. My experiments showed, indeed, that, generally, acid strongly increases and alkali strongly diminishes the coagulation-rate of denaturated albumin. Moreover 0·00005 N HNO_3 increases this rate more strongly than 0·0001 and even 0·0002 N acid so that the increasing effect cannot be explained by the influence of hydrogen-ions; it is evidently due to the formation of acid compounds of denaturated protein the colloidal properties of which change with

the amount of acid contained in them. The same result was also found for the alkali effect. The decrease of coagulation-velocity is in this case not at all proportional to the hydroxyl-ion concentration: 0·001 *N* hydroxyl-ion concentration, in the case of ammonia, produces a similar diminution of velocity as 0·011 *N* hydroxyl-ion concentration in the case of potassium hydroxide. Conversely the like molecular concentrations of ammonia and potassium hydroxide bring about a like decrease of coagulation-velocity so that the decreasing effect of alkali cannot be explained except by the formation of alkali compounds of denaturated albumin having other colloidal properties than the free albumin.

The experiments were made with egg-albumin Kahlbaum, the solutions of which had been dialysed during many days and no longer coagulated on heating.

I. A solution of albumin (containing 2·07 % protein) was boiled and, after cooling, diluted fivefold partly with water, partly with nitric acid. To the solutions obtained potassium sulphate was added to the concentration of 0·083 mol. per litre. The average time of coagulation was found at 75°, in the absence of acid, to be 2700 secs., whilst it was found with 0·00005 *N* acid to be 102 secs., with 0·00010 *N* acid 236 secs., 0·00020 *N* acid 178 secs. But in the presence of 0·01 *N* acid the coagulation proceeded instantaneously.

II. A solution of albumin (containing 3·2 % protein) was boiled and, after cooling, diluted fivefold partly with water, partly with solutions of potassium hydroxide or of ammonia of certain concentrations. Ammonium sulphate was added to the concentration of 0·06 *N*. The coagulation proceeded at 70°, in the absence of alkali, instantaneously, while the average time of coagulation, with 0·0018 *N* ammonia (H-ion concentration = 0·00031 *N*), was 370 secs., with 0·018 *N* ammonia (H-ion concentration = 0·00100 *N*), 760 secs., of 0·2 *N* ammonia (H-ion concentration = 0·00400 *N*), 2200 secs. In the presence of 0·018 *N* KOH (H-ion concentration = 0·01100 *N*) it was 770 secs.

My experiments showed further, that the temperature coefficient of coagulation is in the presence of acid still less than that of coagulation of denaturated albumin in the presence of alkali and even than that of coagulation of arsenic trisulphide. So in a series of experiments, in which dialysed egg-albumin Kahlbaum in the presence of 0·0001 mol. nitric acid per litre was tested, the coagulation time was found to be equal to 237 secs. at 75°, to 400 secs. at 65°, to 720 secs. at 55°. So that the average temperature coefficient was 1·05. In another series, in which the acid concentration was 0·0002 mol. per litre, the coagulation time was: 760 secs. at 75°, 1597 secs. at 55° and 1720 secs. at 50°. The average temperature coefficient of coagulation was, therefore, 1·02. In the presence of 0·018 mol. ammonia per litre this coefficient was found to be 1·12 and in the presence of 0·18 mol. ammonia per litre 1·19.

We might thus suggest, that the colloidal state of acid compounds of

denaturated albumin is nearer to that of typical suspension colloids, while that of alkali compounds is nearer to the state of emulsion colloids. The free denaturated albumin seems to occupy an intermediate position between the alkali compounds and the acid compounds.

Finally, my experiments showed that the acid compounds of denaturated albumin are not identical with the denaturated acid compounds of native albumin, formed in the presence of the same concentration of acid. Its colloidal state is different.

One portion of solution of dialysed egg-albumin containing 2·07 % protein and not coagulating on heating was diluted two and a half-fold with water and boiled up. After cooling, to this protein solution a solution of potassium sulphate and diluted nitric acid were added, altogether in such quantities that the original albumin solution was diluted fivefold. The concentration of acid was 0·0002 mol. per litre, that of potassium sulphate 0·083 mol. The average time of coagulation at 65° was found to be 240 secs., at 55° 365 secs.

The second portion of the same solution of egg-albumin was directly diluted fivefold with diluted nitric acid so that the acid concentration was also 0·0002 mol. per litre. Subsequently the solution was boiled up and cooled; afterwards potassium sulphate was added to the concentration of 0·083 mol. per litre. The average time of coagulation at 65° was found to be 320 secs., at 55° 660 secs.

(7) Influence of the degree of dispersion of denaturated albumin upon its coagulation-velocity.

My experiments relating to the comparison of the time required to produce a certain degree of denaturation of an albumin solution with the time required to bring about the coagulation of the same albumin denaturated by boiling led to an interesting observation which is in accordance with the fact cited above (found by Lillie) that salts lower the degree of dispersion of albumin solutions.

My experiments showed, namely, that the appearance of the definite turbidity of a 0·57 % solution of dialysed egg-albumin Kahlbaum, containing 0·1 mol. potassium chlorate per litre, required on an average 220 secs. at 75°, and 1630 secs. at 71°. However, the same turbidity appeared in 1380 secs. at 75° and in 2020 secs. at 71°, when before adding potassium chlorate the same albumin solution had been boiled and cooled. In both cases the turbidity marked the coagulation of denaturated albumin, but in the first case this coagulation, following the denaturation, proceeded in a lapse of 220 and 1630 secs. (or still more rapidly), while in the second case this coagulation required 1380 and 2020 secs.

It is evident that the colloidal properties of denaturated albumin formed in the solution containing 0·1 mol. potassium chlorate were unlike those of denaturated albumin formed in the solution containing no potassium chlorate, for the former albumin required less time to be coagulated than the latter.

This phenomenon can only be explained by a greater degree of dispersion of the denaturated albumin formed in absence of potassium chlorate. Lillie showed that the degree of dispersion of albumin in the presence of salts is smaller than in the absence of it, even before the denaturation takes place. The denaturation of an albumin of a smaller degree of dispersion leads therefore to the formation of a denaturated albumin which also has a smaller degree of dispersion.

In agreement with the results obtained by Lillie, my experiments showed that the presence of potassium chlorate even in concentration of 0·01 mol. per litre affects the colloidal properties of albumin. So in one of my experiments a 1 % solution of dialysed egg-albumin Kahlbaum (not coagulating on heating) was boiled and, after cooling, diluted with an equal volume of 0·2 *N* KCl. This albumin solution coagulated at 75° on an average in 1380 secs. On the other hand, to the same solution of albumin Kahlbaum KCl was first added to the concentration of 0·01 mol. per litre and the solution was then boiled and after cooling diluted with an equal volume of 0·19 *N* KCl. In this case the protein solution coagulated at 75° on an average in 225 secs., though the concentration of KCl was in both cases the same, namely 0·1 mol. per litre.

The coagulation-velocity of denaturated albumin formed in the presence of potassium chloride was found therefore to be almost sixfold as great as the coagulation-velocity of denaturated albumin formed in the absence of salt. The result obtained corresponds precisely with the value of diminution of osmotic pressure of albumin solution by potassium chloride found by Lillie.

(8) Influence of salts upon the coagulation of denaturated albumin.

In the preceding part of this paper it has several times been pointed out that protein solutions from which salts have been removed by dialysis do not coagulate on heating, but they become coagulable when salts are added to them. Conversely Pauli and his pupil Handovsky affirm that after a very long dialysis the serum (viz. serum-albumin) acquires the property to coagulate on heating without adding salts. Salts added to such serum bring about a rise of its "coagulation temperature," and thiocyanates and iodides make it even heat-uncoagulable [Pauli, 1908; Pauli and Handovsky, 1908, 1909].

These authors call the protein contained in their serum "amphoteric protein," pointing out that it conducts the electrical current not more than distilled water and shows no cataphoresis. It is however a pity that the authors did not define the salt content of their protein, as for instance, by determining the quantity of ash, for salts might not have been dissolved in the water of the serum but adsorbed on the protein particles. Then after the denaturation of albumin they might be liberated anew and produce the coagulation.

At all events, it is impossible to regard the small salt content of "amphoteric protein" of Pauli, as the cause of the possibility of its heat-coagulation, and to suppose that the absence of heat-coagulation of dialysed protein solutions which has been observed by all other authors is due to the incompleteness of the dialysis. Indeed, the addition of salts to the "amphoteric protein" could cause only a rise of coagulation temperature, and only thiocyanates and iodides (normally absent in natural liquids) made, in the experiments of Pauli, the heat-coagulation of serum impossible.

In order to render the influence of salts upon the coagulation of denaturated protein clear, I made experiments with egg-albumin and serum-albumin Kahlbaum. We consider first the influence of salts upon the coagulation of egg-albumin.

The solutions of egg-albumin (5–10 %) were usually centrifuged and filtered. They had a slight alkaline reaction (litmus) and were almost water-clear. After drying a part of the solutions in vacuum and afterwards in the drying-stove the dry albumin was found to contain about 3 % ash, of which the most part (2 %) was water-soluble (KCl, NaCl, K_2SO_4, K_2CO_3). The solutions coagulated on heating.

The dialysis was executed by means of the thinnest parchment paper which could be purchased. If the dialysis was prolonged an antiseptic was added to the solutions tested and the dialyser was placed in a covered vessel. The solution layer in the dialyser never exceeded $\frac{1}{2}$ cm. in depth.

After 24 hours of dialysis (distilled water was changed each two hours) the protein solutions usually became neutral (litmus) in reaction and then lost the property of coagulating on heating (if the protein-content was relatively great, *e.g.* 10 %) or they lost this property only after two or three days of dialysis (if the protein-content was small).

The dialysed solutions were usually centrifuged and filtered anew till they became water-clear. The content of protein and of mineral substances in the solutions (which did not coagulate on heating) is set out below.

Number of tested solutions	Original concentration of albumin	Duration of dialysis	Concentration of albumin after dialysis	Content of mineral substances in dry albumin after dialysis	Content of soluble mineral substances in g. per 100 cc. of solution after dialysis
	%	days	%	%	
1	10	1	7·5	1·8	0·05
2	4·9	2	3·29	1·1	0·02
3	4·1	5	2·07	1·2	0·01

In order to answer the question whether solutions of egg-albumin which no longer coagulate on heating would acquire the property to coagulate anew by a still more prolonged dialysis, solutions 1 and 3 were employed for a further dialysis.

To solution No. 1 an excess of chloroform was added as an antiseptic. After 10 days of dialysis at 20° this solution showed heat-coagulation anew.

But it acquired an unpleasant scent and, in spite of the chloroform, contained many bacteria. The heat-coagulation was evidently due in this case to organic acids or salts which had been formed by the bacteria. Indeed, after a dilution of the solution with water the heat-coagulation disappeared anew, but it reappeared if some ammonium sulphate was added to it.

To solution No. 3 an emulsion of melted thymol in water was added as an antiseptic. The dialysis lasted three weeks. The solution contained after such dialysis 0·99 % albumin. The dry albumin (drying in vacuum) was found to contain 0·8 % ash of which about a half was soluble in water. The solution contained 0·008 % dissolved mineral substances and coagulated on heating. An unpleasant scent and bacteria were however absent.

The albumin solution thus obtained showed the required degree of coagulation (turbidity like that of the ground glass, see above) at 75° in 27 secs. After dilution of the solution with water to double the volume, the same degree of coagulation was observed at 75° in 700 secs., at 80° in 370 secs., at 70° in 1260 secs. The striking increase of coagulation-time after a relatively inconsiderable dilution with water indicates that the very small amount of salts contained in the albumin, which was sufficient to bring about the coagulation of protein in 27 secs. at 75°, after the dilution with water, could no longer produce the coagulation in this time. At the same time, the small temperature coefficient—1·12 and 1·14 per temperature-rise of 1°—showed that the coagulation-velocity, after the dilution with water, was smaller than the denaturation-velocity.

When this albumin solution was diluted fivefold with water, it ceased to coagulate even on boiling, whereas it coagulated easily if some ammonium sulphate was first added to it. Similarly it coagulated if after boiling and cooling this salt was added to it.

It seemed, therefore, that the heat-coagulation of denaturated egg-albumin has two optimal concentrations of salts present in the solution. Namely the coagulation on heating takes place when this concentration is more than 0·1 % (viz. about 0·01 *N*), it ceases to be observed when this concentration is between 0·01 and 0·05 % (viz. about 0·001 and 0·006 *N*), it appears anew when this concentration diminishes to 0·008 % (viz. about 0·0008 *N*), and, finally, it ceases again to appear when the salt-concentration becomes about 0·001 % (viz. about 0·0001 *N*).

Similarly, two most favourable salt-concentrations, viz. two coagulation-zones, were observed also on the other colloids, as for example, on lecithin and cholesterol [Porges and Neubauer, 1908]. Nevertheless, my experiments could not confirm the existence of any coagulation-zones in the case of heat-coagulation of protein.

They showed that a gradual increase of salt-content produces, instead of a retardation or a cessation of heat-coagulation, an acceleration of this coagulation of protein. In my experiments, the above solution of albumin containing 0·008 % dissolved mineral substances was diluted with an equal

volume of solution of potassium chlorate of various concentrations and the heat-coagulation time was determined by the previous method.

When potassium chlorate was substituted by potassium thiocyanate, the phenomenon remained the same, and only very strong concentrations of this salt (2·5 mol. per litre) brought about a diminution of coagulation-velocity. A suppression of heat-coagulation by adding KCNS could never be observed. At a concentration of KCl of 0·003 *N* the temperature coefficient was found to be equal to 1·17; and only at the concentration of 0·01 *N* did this coefficient attain the value of the normal denaturation coefficients 1·35–1·40, and the denaturation began to proceed more slowly than the coagulation of the denaturated albumin. The same was also observed, when a sufficient amount of KCNS (0·1 mol. per litre) was added to the solution. On the other hand an excess of this salt (2·5 mol. per litre) retards the coagulation anew and the denaturation begins to proceed more rapidly than the coagulation (the temperature coefficient = 1·04).

The albumin contained in the solution which had been dialysed during four weeks is therefore not identical with that contained in the solution which had been dialysed during 5–7 days. After denaturation the former albumin coagulates more easily under the influence of salts than the latter. The degree of dispersion of the former is probably smaller than that of the latter. At all events the very prolonged dialysis evidently alters the original albumin. Both albumins are however "amphoteric" (see Pauli), for neither the one nor the other showed in my experiments cataphoresis by an electrical current of 110 volts; but both migrated to the anode after denaturation (according to the increase of alkalinity of the solution).

We pass now to the experiments with serum-albumin.

A 5 % solution of serum-albumin Kahlbaum was centrifuged and filtered. The slightly alkaline solution obtained was very faintly opalescent, coagulated on heating and contained 3·07 % dry residue of which 0·25 % was mineral matter.

The dry protein therefore contained 8·4 % mineral substances. The soluble part of the latter consisted of sodium chloride, potassium chloride, sodium carbonate, etc., the insoluble part was also insoluble in acids (SiO_2?).

The albumin solution obtained was dialysed during 20 hours. After the dialysis it was centrifuged and filtered anew. It reacted neutral (litmus) and no longer coagulated on heating, but only became opalescent. It now contained 2·4 % dry residue, of which 0·07 % was mineral and 0·05 % water-soluble. The dry albumin contained now about 3 % mineral substances.

After adding salts to this albumin solution heat-coagulation could again be produced on heating.

The high temperature coefficient of heat-coagulation (= 1·55) after adding potassium chloride showed that 0·1 mol. per litre of this salt was already

sufficient to make the coagulation of the denaturated albumin proceed more swiftly than the denaturation.

In fact, heating the solution of dialysed serum-albumin after a preliminary boiling and cooling and the subsequent addition of potassium chloride to a concentration of 0·1 mol. per litre, showed in my experiments that the coagulation of the denaturated albumin at 70° required on the average only 35 secs., at 68° 50 secs., at 65° 90 secs., etc. On the other hand the same concentration of potassium thiocyanate only brought about a coagulation which proceeded slower than the denaturation, and the temperature coefficient was found in this case to be low. Only between 65° and 70° did the denaturation-velocity begin to be greater than the coagulation-velocity and the temperature coefficient increased, while the coagulation-time became equal to that observed in the presence of potassium chloride.

Further dialysis renders the solution of serum-albumin heat-coagulable again, and even after 6 weeks of dialysis the solution coagulated on heating. At the same time even after 5 days of dialysis the filtered solution contained 1·28 % dissolved solids, of which 0·02 % was mineral; the dry albumin therefore now contained 1·4 % mineral substances (half of which was soluble in water). Further dialysis however altered this salt-content of the albumin solution very little.

Similarly to the solutions of egg-albumin those of serum-albumin, when dialysed during a long time, had a low temperature coefficient of heat-coagulation, the denaturation proceeding evidently more rapidly than the coagulation of the denaturated albumin. The heat-coagulation at 70° required in my experiments on the average 840 secs. and at 80° 510 secs.; the temperature coefficient per rise of 1° was thus found to be equal to 1·05.

Further, a very small concentration of salts (KCl or KCNS) was found in my experiments to accelerate the coagulation of denaturated serum-albumin, and the increase of salt concentration accelerated it so strongly that its velocity became greater than the denaturation-velocity, and the temperature coefficient was found high again and like that observed for solutions of serum-albumin which had not been dialysed (1·69).

The results obtained in my experiments on serum-albumin are therefore like those obtained on egg-albumin. The difference is only quantitative. The solution of serum-albumin loses its mineral substances considerably more readily by dialysis than the solution of egg-albumin. After two days of dialysis the solution of serum-albumin has lost the greater part of its mineral matter; and after 5 days only one-twelfth of this remains in the solution.

Further, the disappearance of heat-coagulation of serum-albumin is observed when the salt-content of the solution is between 0·03 and 0·07 %, while the absence of heat-coagulation of egg-albumin takes place when this content is between 0·01 and 0·05 %. On the other hand the addition of salts

(even of potassium thiocyanate) to the solutions of both albumins, made coagulable on heating by a very prolonged dialysis, cannot render them incoagulable on heating anew.

We have thus to conclude that in both cases albumin is altered by a very prolonged dialysis in such a manner that, after denaturation it shows a greater susceptibility to salts than before. In what however this alteration consists, is still unknown. It is probable that the degree of dispersion of both albumins became smaller by lapse of time, or that a small part of the salts, contained in albumin solutions, are chemically united with the native albumin and the prolonged dialysis decomposes such salt compounds. Free albumin, after denaturation, is in this latter case more susceptible to salts than its unions with salts.

It was formerly pointed out that Pauli and Handovsky affirm that they have found the addition of salts to "amphoteric albumin" to bring about an increase of its "coagulation-point." This observation contradicts the results of my experiments which showed, as mentioned, an increasing effect of salts upon the coagulation-velocity of albumins. But nevertheless, according to Pauli and Handovsky, my experiments showed that potassium thiocyanate and iodide in strong concentration (more than 1 mol. per litre) diminish the coagulation-velocity of denaturated albumin and in the case of serum-albumin can even make it incoagulable on heating when they are present in almost saturated solution. This phenomenon can be explained by the formation of chemical compounds of these salts with denaturated serum-albumin. This albumin is still soluble in a strong and boiling solution of potassium thiocyanate [Pauli and Handovsky, 1908][1].

Moreover, the results of my experiments concerning the influence of dilution with water upon the coagulation-velocity of denaturated albumin and particularly upon that of denaturated serum-albumin also contradict the result obtained by Pauli and Handovsky. These authors point out that an increasing concentration of protein produces a considerable rise of the coagulation-point. My experiments show however that dilution of heat-coagulable albumin solutions which had been very long dialysed diminishes the coagulation-velocity and a sufficiently strong dilution makes it so small as to render it inaccessible to observation.

What is the cause of all these contradictions? Could it not lie in that conjuncture which had taken place in the experiments of Pauli and Handovsky, who, before determining the coagulation-point of serum dialysed 6 weeks, left it to settle during 3–5 months [1908, pp. 416–8]? It is possible that during such a long time the serum-albumin was altered under the influence of water and the oxygen of the air. In one of my experiments, at least, a solution of serum-albumin, having been dialysed during 6 weeks and coagu-

[1] The degree of dispersion of compounds of albumin with thiocyanates and iodides is probably only greater than that of free denaturated albumin, a sufficient amount of ammonium sulphate making them coagulable anew.

lating on heating lost this property and became slightly alkaline after standing during one year (thymol as antiseptic).

We now pass to the experiments on the influence of salts upon the coagulation-velocity of denaturated albumin dialysed during some days. In these experiments albumin solutions were dialysed until they no longer coagulated on heating without the addition of salts.

We have seen above that increase of salt concentration accelerated the coagulation of denaturated albumin which had been very thoroughly dialysed and coagulated on heating. By virtue of low temperature coefficients we have, indirectly, concluded that in this case of heat-coagulation we have been dealing with the coagulation of denaturated albumin. To investigate the influence of salt concentration upon the coagulation of denaturated albumin not coagulating on heating, without the addition of salts, is evidently much simpler. The solutions of dialysed albumin were in my experiment first boiled up and, after cooling, mixed with salt solutions of a certain concentration; the albumin solutions obtained were then tested at certain temperatures in the thermostat. I will cite here an example of my experiments.

A solution of egg-albumin Kahlbaum containing 2·85 % protein and not coagulating on heating (owing to dialysis during 5 days) was boiled up, and, after cooling, diluted fivefold with solutions of ammonium sulphate of various concentrations so that the albumin solutions obtained contained 0·025, 0·05, 0·1 and 0·15 mol. ammonium sulphate per litre. At 47° the times required to produce the standard turbidity were found to be respectively equal (on an average) to 10,600 secs., 1620 s., 40 s. and 17 s.

The experiment cited shows that the dependence of the coagulation-rate upon the salt concentration of the solution is not like the dependence of the adsorption of salt upon the salt concentration. The coagulation is therefore not a simple adsorption of electrolytes whose ions electrically discharge the colloidal protein particles.

According to the well-known adsorption-isotherm $\frac{x}{m} = ac^{\frac{1}{n}}$, the adsorbed quantity of dissolved substance increases more slowly than the concentration of this substance in the solution. In my experiment however the increase of the salt concentration of twofold was accompanied by an increase of coagulation-velocity of ten- or fourteen-fold.

As mentioned above Hardy showed that the denaturated protein can be precipitated by adjusting the reaction so as to render the particles iso-electric with the solution; but when the protein is made negative the valency of the cation is of importance for its precipitation. It has already been pointed out that the colloidal particles in the solution of egg-albumin Kahlbaum, which has been dialysed for some days (4–6 days), are iso-electric with the solution (no cataphoresis was observed) and that they become negative after denaturation. According to Hardy we should therefore expect that the cation only would be important in the coagulation of denaturated protein of the albumin solution in question. My experiment showed however that not only cations

but also anions are of importance in the process. The results of experiments are set out below.

I. The material used was egg-albumin Kahlbaum, a solution of which had been dialysed for 6 days (albumin-content 2·8 %). It was boiled and after cooling diluted with solutions of salts (fivefold). The average times of coagulation at 75° were found to be 2100 secs. in the presence of 0·1 *N* KCl, 1450 secs. at 0·1 *N* NH_4Cl, 1180 secs. at 0·1 *N* NaCl, 1057 secs. at 0·1 *N* LiCl, 1287 secs. at 0·003 *N* $BaCl_2$, 1320 secs. at 0·003 *N* $MgCl_2$, 1080 secs. at 0·1 *N* $FeCl_3$, 2540 secs. at 0·1 *N* $AlCl_3$. In the presence of 0·02–0·1 *N* $BaCl_2$ or $MgCl_2$ the coagulation proceeded instantaneously. In the presence of 0·003 *N* $FeCl_3$ very slowly.

II. The same solution of egg-albumin. The average times of coagulation at 75° were found to be 215 secs. in the presence of 0·1 *N* K_2SO_4, 826 secs. at 0·1 *N* potassium tartrate, 1070 secs. at 0·1 *N* KNO_3, 1363 at 0·1 *N* KCl, 1406 secs. at 0·1 *N* KBr, 1826 secs. at 0·1 *N* KI and 3826 secs. at 0·1 *N* KCNS.

Therefore, the cations act upon the coagulation-velocity of denaturated albumin not at all proportionally to their valency; the tervalent cations (Al, Fe) produce an acceleration of this velocity almost equal to that produced by the univalent ions. The bivalent cations (Ba, Mg) are however strikingly active, producing in the concentration of 1/33 of the univalent ions an almost equal acceleration of coagulation-velocity. In the single series of cations of equal valency the accelerating effects are as follows: Li > Na > NH_4Cl > K; Ba > Mg and Fe > Al. In all cases the temperature coefficients are relatively great (1·1), and then the suggestion emerges that the different effects of cations are due to some chemical influence produced by these cations. The latter could, for example, form adsorption compounds with denaturated protein. The yellow colour of the precipitate brought about by ferric chloride directly confirms this suggestion. At all events, the observed effect of cations shows that the coagulation of denaturated albumin cannot simply be regarded as a process of electrical discharge of protein particles by the ions. The same results also from the observed effect of various anions.

In spite of the slight negative charge carried by the particles of denaturated albumin the anions employed can be placed in the following series:

$$SO_4 > \text{tartr.} > NO_3 > Cl > Br > I > \text{Thiocyanate.}$$

Both the series of ions (that of univalent cations and that of anions) are the well-known lyotropic series of ions which were found for all precipitations of emulsion-colloids.

The observed difference in effect of single anions becomes still sharper when the solution of denaturated albumin is made more alkaline by adding potassium hydroxide. In this case, as already mentioned, protein certainly forms compounds with alkali, which are definitely nearer to the emulsion-colloids than the free denaturated albumin (see above). In my experiments

the coagulation of denaturated albumin proceeded at 75° instantaneously when the solution contained 0·001 mol. per litre potassium hydroxide and 0·5–1 mol. K_2SO_4; whilst the average time of coagulation was 180 secs. in the presence of the same quantity of alkali and 1 mol. KCl, it was 1220 secs. with 1 mol. KNO_3. In the presence of 1 mol. of KI or KCNS the coagulation did not appear at all. KCNS produces a coagulation only when it saturates the solution.

As already mentioned, dialysed albumin shows, after denaturation, a cataphoresis to the anode, so that it is probable that the reaction of the solution becomes in this case slightly alkaline (see above, Hardy). But it was pointed out that even very slight concentrations of alkali can cause the formation of compounds with denaturated protein. It is therefore possible that the lyotropic series of ions found is observed only when the coagulation of alkali compounds of protein is investigated. In order to study the influence of ions upon the coagulation of alkali-free albumin, I added to the albumin solution, after denaturation, nitric acid to the concentration of 0·0002 mol. per litre. This concentration is not sufficient for the formation of acid-compounds of albumin (see above). Nevertheless the lyotropic series of anions was perceptible even in this case.

If in my experiments the acid concentration increased to 0·004 mol. per litre and above, the denaturated albumin formed compounds with acid (see above) and the lyotropic series of anions became indefinite, but the valency of the anions came to the foreground. Potassium sulphate, for instance, was found to accelerate the coagulation considerably more strongly than potassium chloride; on the other hand, according to its lyotropic property (the series is in this case inverse) potassium thiocyanate had a still greater effect upon the coagulation than potassium sulphate. The results obtained by Hardy were confirmed only in part.

The study of the influence of salts upon the coagulation-velocity showed generally that the process of coagulation of denaturated albumin is not simply a pure physical phenomenon of discharge of colloidal protein particles owing to an adsorption of electrolytes (ions), but that it is, at least partly, a chemical phenomenon, in which not only electrical properties but also chemical properties of salts are significant.

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LXXI. THE SYNTHESIS OF GLYCINE FROM FORMALDEHYDE.

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For the purpose of some work on which we are engaged, it became necessary to secure a considerable quantity of glycine. The published methods for the preparation of this compound are unsatisfactory and difficult to carry out. Glycine was first obtained by Braconnot [1820] by the hydrolysis of gelatin with dilute sulphuric acid or baryta. Perkin and Duppa [1858] prepared it by treating bromoacetic acid with ammonia. It has also been prepared by treating chloroacetic acid with ammonia or ammonium carbonate. This method of preparation is dealt with in subsequent papers by Heintz [1862], Nencki [1883], Mauthner and Suida [1888, 1890]. When prepared by either of these methods, the glycine must be isolated by means of the copper salt, and the yield never exceeds 20 % of the theoretical.

Gabriel and Kroseberg [1889] obtained an almost theoretical yield of glycine by hydrolysing ethylphthalylglycine with hydrochloric acid. This method from an economic standpoint is unsuitable for the preparation of large quantities of glycine.

Eschweiler [1894] states that he obtained glycine in almost theoretical yield by treating methylene cyanohydrin with a large excess of ammonia. Here the difficulty is the preparation of the cyanohydrin.

Attempts have been made by us to devise a direct method for the synthesis of glycine from formaldehyde. Methylene-aminoacetonitrile can be obtained in a yield of 60 % of the theoretical by the condensation of formaldehyde (2 mols.) with ammonium cyanide as shown by Klages [1903], thus:

$$2H\,.\,CHO + NH_4CN = CH_2 : N\,.\,CH_2\,.\,CN + 2H_2O.$$

Klages' method is carried out as follows. Finely powdered ammonium chloride (360 g.) is added to 40 % formaldehyde (1000 g.), in a wide-necked glass jar, cooled to 5° in a freezing mixture, the solution being stirred by means of a strong electric turbine. Potassium cyanide (440 g.), dissolved in water (600 cc.) is slowly run in during 3 hours, the temperature being kept during this time below 10°. It may be mentioned that commercial 96 % cyanide, which consists of a mixture of potassium and sodium cyanide, may be employed. When half the cyanide solution has been added, the ammonium chloride will have completely dissolved. The remainder of the cyanide and at the same time glacial acetic acid (250 cc.) is then dropped in. When the whole of the

acetic acid has been added methylene-aminoacetonitrile commences to separate in glistening white crystalline flocks. The solution is now stirred with a turbine for 2 hours and the crystalline mass collected on a Buchner funnel. After washing with cold water and drying on a porous plate, the yield is about 280 g. or 60 % of the theoretical. It melts at 129°.

When the nitrile is hydrolysed with hydrochloric acid, the plan adopted by Klages, considerable difficulty is encountered in separating the glycine from the ammonium chloride produced simultaneously. It was found, however, that the nitrile could be easily hydrolysed by boiling it with a concentrated (40 %) solution of barium hydroxide.

The nitrile (10 g.) is added in small portions at a time to a boiling 40 % solution of barium hydroxide (100 cc.) in an open beaker. The mixture is boiled until no more ammonia is evolved, the volume of the liquid being kept approximately constant by the addition of water from time to time. The total period of boiling is 3 hours. From the solution, which now contains the methylene derivative of glycine, the barium is precipitated with sulphuric acid, the filtrate and washings from the barium sulphate are acidified until 3 % of sulphuric acid is present, and the liquid is boiled in an open beaker until no more formaldehyde is given off, which usually requires about 4 hours. The reaction proceeds as follows:

$$CH_2(NCH_2)COOH + H_2O = CH_2(NH_2)COOH + HCHO.$$

The sulphuric acid is removed by the addition of barium hydroxide, and the filtrate and washings containing the glycine are decolorised by boiling with norit. Traces of barium are removed by the addition of the requisite quantity of standard sulphuric acid. The filtrate is concentrated on a water-bath and the glycine which separates is recrystallised from alcohol. The yield is 90 % of the nitrile employed or taking the yield of the nitrile as 60 % of the theoretical, the yield of glycine from formaldehyde is 54 % of the theoretical.

This is the highest yield of glycine yet recorded as a result of its direct synthesis from formaldehyde. Our procedure has many advantages over the methods previously published, one being that the glycine is obtained uncontaminated with inorganic salts which are very troublesome to separate. Indeed methods of separating these salts have formed the subject matter of patents [Farbw. Meister, Lucius and Brüning, 1903; Siegfried, 1907].

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LXXII. AN INVESTIGATION OF THE CHANGES WHICH OCCUR IN THE PECTIC CONSTITUENTS OF STORED FRUIT.

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An accurate method of estimating pectin in dilute solution by precipitating as calcium pectate, has recently been established. The method has been successfully applied to the estimation of pectin in some fruit juices and by the application of this method it was proved that a complete extraction of the soluble pectin of apples can be effected by a process of continuous washing out with water.

As a result of this preliminary work it was found possible to follow the changes which take place in the pectic constituents of apples kept in cold and ordinary storage and to determine if the improved keeping properties of the apples in cold storage is accompanied by any marked difference in pectin content.

The soluble pectin probably develops from an insoluble pectic substance contained in the cell wall, which is left behind in the pulp residues after the aqueous extraction of the soluble form. This insoluble pectin corresponds to the protopectin of Fellenberg [1918], and to the pectose of earlier investigators.

Estimations of the soluble pectin were systematically carried out at regular intervals throughout the period of storage. The apples chosen for the experiments were very different, Lane's Prince Albert, a hard and acid cooking apple, and the soft, sweet Cox's Orange Pippin. Equal quantities were stored in cupboards at the ordinary temperature, and in a refrigerator at 60° C.

In view of the possibility that the time of harvesting might influence the keeping properties of the fruit, the estimations were carried out on three pickings, which were subsequently kept in cold and ordinary store. Batches were gathered three weeks earlier than the usual harvesting, at the normal time in early September, and the third picking was left on the trees till October.

Estimation of the Soluble Pectin.

The following is a summary of the process of extraction and estimation of the soluble pectin; a more detailed account of the method has recently been published [Carré and Haynes, 1921].

Samples of ten apples were cut up, freed from skin and core, and thoroughly mixed. Fifty grams were used for each estimation throughout the season.

After killing the cells, by freezing the weighed portion of the material in an efficient freezing mixture, the extraction of the soluble pectin was carried out by washing with water and pressing out in a small hand press. These processes were repeated until all the pectin was extracted. The completeness of the extraction was verified in all cases by testing the last washings of the pulp for pectin by precipitation as calcium pectate.

The dilute juice was filtered through fluted papers, to remove insoluble matter and disintegrated cell substance. It was then boiled to destroy enzyme action, and after cooling, the solution was made up to a known volume.

Aliquot portions were taken for each estimation, the quantity used depending on the concentration of the pectin solution. It was found advisable to make a rough preliminary determination of the amount of pectin contained in the juice solution, since, as the fruit approached maturity, the development of the soluble pectin from an initial negligible quantity to as much as 0·8 % necessitated a careful adjustment of the conditions of estimation.

The best results were obtained by using a volume of solution yielding 0·02–0·03 g. of calcium pectate.

For this weight of pectin, 100 cc. $N/10$ soda were used for the hydrolysis, and after 24 hours' standing the pectin was precipitated as calcium pectate by acidification with 50 cc. of $N/1$ acetic acid, and subsequent addition of 50 cc. $M/1$ calcium chloride. During the later period of storage in the case of both Cox and Lane apples, it was observed that, although the pectin content did not appreciably differ and the conditions of experiment were exactly as before, the coagulation of the calcium pectate became ill defined, and the precipitate was slimy and difficult to wash and filter. It was found however that a satisfactory coagulation could again be obtained by using double the amount of $N/10$ soda previously found adequate for hydrolysis. The larger amount of soda was found to be necessary as long as the supply of fruit was available, and it seems reasonable to conclude from these observations that some change occurs in the state of aggregation of the pectin sol as the fruit develops, or possibly an alteration may take place in the constitution of the pectin.

The Development of Soluble Pectin.

The accompanying graphs (Figs. 1 and 2) are representative of the results obtained from the estimations. Fig. 1 illustrates the development of soluble pectin in cold and ordinary storage, and Fig. 2 shows the very close similarity in the development of the soluble pectin in all three pickings of apples.

The experiments indicate that in the early stages of the maturation of the apple there is no soluble pectin, but it gradually develops as ripening proceeds till it attains a maximum amount when the fruit reaches its fully ripe condition. The Cox apples ripened earlier than the Lane, and in both cases the fully ripe state was found to be coincident with the greatest amount of pectin developed. The maximum pectin content was maintained in all cases for

about 4 weeks, during which the apples were in their prime condition. This period was followed by a condition of general softening and a sudden drop in the pectin content was found to accompany the change of condition in the fruit. This softening process is the concluding phase in the apple's life, and it leads eventually to a state of physiological breakdown or of general decay brought about by fungal or bacterial action. During the whole softening

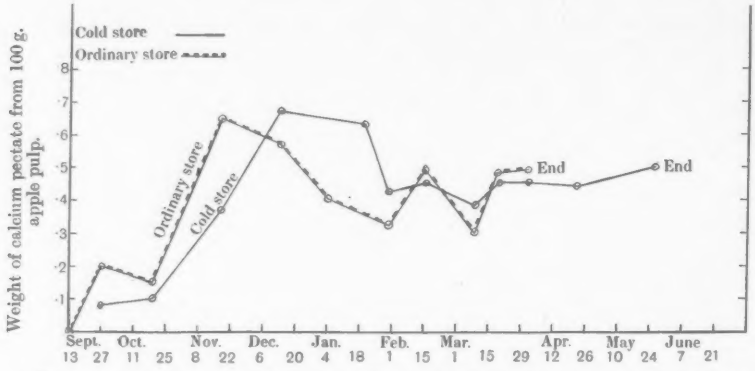
Cold store
Ordinary store
Weight of calcium pectate from 100 g. apple pulp.
Ordinary store
Cold store
End
End
Sept. Oct. Nov. Dec. Jan. Feb. Mar. Apr. May June
13 27 11 25 8 22 6 20 4 18 1 15 1 15 29 12 26 10 24 7 21

Fig. 1. Development of soluble pectin in cold and ordinary storage (Lane—1st picking).

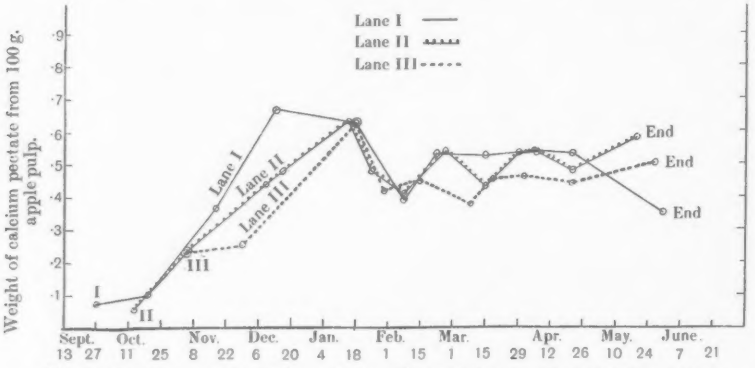

Fig. 2. Development of soluble pectin in cold stored Lane's picked at different times.

period the soluble pectin content showed considerable variations. In order to ascertain if these variations could be accounted for by differences in the condition of the fruit estimations of pectin were made on sets of apples in different states of softness, each set being carefully selected to be as uniform as possible. The results obtained show that there is a marked decrease in pectin as the fruit softens. The following experiments illustrate this point:

	Hard sample	Soft sample
Weight of soluble pectin from 100 g. ...	0·51 g.	0·42 g.

It may therefore be concluded that the fluctuating values obtained for the soluble pectin are to be attributed to the great variability which was observed in the state of maturity of the individual apples taken for each estimation, and it follows that in samples of ten taken for each separate determination a large sampling error might easily result. It is proposed during the next season to carry out estimations on each of the individual apples and to calculate the probable error due to sampling.

The routine estimations showed that apples contained varying but appreciable amounts of pectin as long as supplies of fruit were available, and in no case where sound apples were examined did the pectin wholly disappear. On the other hand when diseased apples were examined there was found in most cases to be a complete absence of soluble pectin.

Effect of Cold Storage on the Development of Soluble Pectin.

The curves showing the development of pectin in cold and ordinary stored fruit (Fig. 1) are very similar, the only effect of the low temperature being to prolong the period of ripening. The maximum point of soluble pectin content occurs six weeks to two months later in the cold stored fruit.

After the maximum was passed and during the breakdown of the fruit the pectin content of the cold and ordinary stored fruit was found to be practically identical as long as samples of both were available for comparison.

It was possible to carry out these comparisons up to the end of April on the cold and ordinary stored Lane's, but the Cox's Orange were so badly affected by Bitter Pit that the supply of ordinary stored fruit was exhausted by the end of December, soon after the maximum point had been reached. The greatly prolonged life of the cold stored Cox's as compared with those kept in ordinary storage was due to the effect of cold in retarding the progress of disease in the fruit.

If the results of the estimations of soluble pectin developed in cold and ordinary store during the normal life of the apple are added together, the quantity is found to agree very closely for apples of the same kind whether they have been picked at different stages of maturity or kept under different conditions of temperature.

Total of (12) estimations during six months.

	Cold stored	Ordinary stored
Lane I[1]	4·05 g.	4·07 g.
Lane II	3·99	4·03

Total of (7) estimations during three months.

Cox I	3·01 g.	3·07
Cox II	2·77	2·82

	Picking		
	1	2	3
Lane	4·29	4·11	4·45
Cox	4·95	4·70	4·64

[1] = the numerals indicate the different pickings of the apples.

This agreement is too close to be merely fortuitous, but it is impossible to attempt any complete interpretation at present. It may however be inferred from the foregoing results that a definite amount of soluble pectin is produced during the process of ripening and that it is subject to secondary change during the later stages of maturation.

Estimations of protopectin which are described below, throw some further light on the subject.

Estimation of Protopectin.

With a view to examining the pectin changes in the apple more thoroughly it became necessary to devise a quantitative method of estimating the insoluble pectin constituent or protopectin[1], as it will be called in the following account, and so to obtain some idea of the relations between the soluble and insoluble forms.

The method adopted was to treat the residue after extraction of the soluble pectin with 100 cc. $N/20$ HCl in an autoclave for about an hour at a temperature of 110°. The material became thoroughly disintegrated during the process and the protopectin was converted into the soluble form, presumably by a process of hydrolysis. It was easily washed out with very little pressing, and the extract was estimated by the calcium pectate method. That the treatment removes all the insoluble pectic substances capable of being transformed by the acid was proved by repeating the autoclaving with a similar amount of $N/20$ HCl in which case no soluble pectin was found in the second extract. Subsequent treatments with greater concentrations of acid $N/5$ and $N/1$ also failed to produce any further trace of pectin.

To test the accuracy of the method equal amounts of apple pulp were taken, and the soluble pectin in them was extracted and estimated. The residues were treated as described above in order to convert the protopectin into soluble pectin, and the extracts thus obtained were also estimated. The following results illustrate the measure of agreement in the amounts obtained from two samples of uniform material. Both protopectin and soluble pectin are estimated in terms of the calcium pectate obtained from them.

Weight (g.) obtained from 100 g. of pulp.

	Soluble pectin	Protopectin	Total pectin (Protopectin and pectin)
(*a*)	0·43	0·36	0·79
(*b*)	0·385	0·45	0·835

It has been found possible to estimate the total pectin directly from the apple pulp without preliminary washing out of the soluble form.

[1] The adoption of this name for the hydrolysable pectin present in the cell wall implies that this is the precursor of soluble pectin. While there is much reason to believe that this is so, the fact cannot be regarded as finally established, and the present nomenclature must therefore be considered provisional.

Apple Sample I from Lane.

(Weight obtained from three similar samples of 100 g. of apple.)

	Picking		
	1	2	3
Soluble pectin ...	—	—	0·348
Protopectin ...	—	—	0·094
Total ...	0·404	0·418	0·442

Apple Sample II from Cox.

Soluble pectin ...	—	—	0·52
Protopectin ...	—	—	0·24
Total pectin	0·71	0·71	0·76

The amounts estimated in this way are in fairly close agreement with the total arrived at by adding the weights obtained by separate estimation of the soluble and insoluble pectin. The slightly lower values obtained in both these examples where the total pectin is extracted from the pulp may be entirely due to experimental error, but it is possible that some change may be brought about by the acids and salts contained in the juice.

Estimation of Protopectin of apples kept in cold and ordinary store.

During the latter half of the period of storage this method was systematically used in a preliminary examination of the protopectin content of apples. Estimations were carried out on the residue of the weighed sample from which all the soluble pectin had been extracted. The residue was then autoclaved with acid as described above, and the resulting soluble pectin precipitated as calcium pectate and weighed. In this way the weight of protopectin and soluble pectin was determined in a given sample of apple.

The results obtained are of necessity incomplete, and the following account must be regarded as in the nature of a preliminary survey.

The protopectin determinations were made after the maximum soluble pectin content had been passed and the protopectin shows the tendency to fluctuate which was observed in the case of the soluble pectin.

The following estimations of the protopectin content of hard and soft apples show that there is less in the softer fruit. As the samples contained individuals differing greatly in their degree of maturity these fluctuations may be attributed, as in the case of the soluble pectin, to the variation of individual apples in each set taken for estimation.

Weight of protopectin in 100 g. of samples	Hard sample	Soft sample
A. Lane II ordinary stored	0·45	0·35
B. Lane II cold stored	0·28	0·18

A series of estimations of pectin and protopectins carried out at regular intervals on the same apples, showed that a very definite relationship exists between them and that the changes in the two constituents tend to be equal

and opposite in amount. The accompanying graph (Fig. 3) is typical of the results obtained:

The foregoing results show that after the maximum soluble pectin content is passed the quantity of soluble pectin tends to decrease and that the decrease is roughly proportional to a simultaneous increase in the insoluble pectin. On the other hand both the protopectin and soluble pectin have been shown to decrease markedly in soft over-ripe fruit as compared with harder less ripe fruit.

These observations seem to indicate that there is a balance between the protopectin and soluble pectin during the normal life of the apple, but that in the later stages of breakdown the total pectin tends to decrease. On the other hand there is some evidence to show that the total pectin content tends to *increase* as the apple ripens. The following estimations were carried out

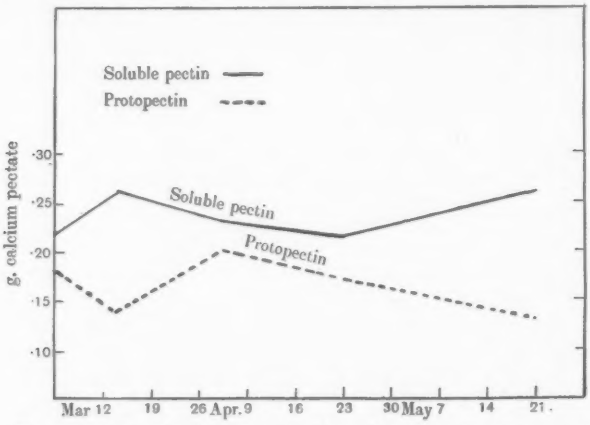

Fig. 3. Relationship of pectin and protopectin in Lane II ordinary store.

on cold stored "Cox's" when the apples were in the early stages of their development and during the period of fluctuation when the fruit was beginning to get over-ripe.

	Soluble pectin	Protopectin	Total
Early unripe state (Sept. 20) ...	0·05	0·48	0·53
Fully ripe state (Feb. 16–Mar. 16)[1]	0·65	0·36	1·01

[1] These figures represent the mean of the weights obtained during this period.

These results need confirmation but appear to show that in the few weeks following the picking of the apples the total pectin increases rapidly owing to the marked development of soluble pectin. The change in the protopectin content is much less pronounced and is insufficient to account for the increase of the soluble form. It may therefore be inferred that the total increase in pectin is due to a third source of pectin, which either gives rise to soluble pectin directly, or is transformed into it through the intermediate state of protopectin.

THE FACTORS CONTROLLING THE DEVELOPMENT OF SOLUBLE PECTIN.

The following investigations were made to obtain some information as to whether the production of soluble pectin is due to enzyme activity.

Samples of apple which had been previously killed by freezing were crushed with sand and thoroughly washed and pressed free from soluble pectin. The residues were left with water and a little thymol added to prevent bacterial action. At the end of a week the residues were again pressed out and tested for soluble pectin. In all cases examined pectin was found to have developed. The samples were put back in water and the process repeated every week till no more pectin was found to have developed. The following examples carried out on ordinary stored "Cox's" illustrate the results so obtained.

WEIGHT OF SOLUBLE PECTIN DEVELOPED FROM 100 G. OF APPLE PULP.

	A Cox III Ordinary stored	B Cox II Ordinary stored
1st week	0·22	0·30
2nd week	0·35	0·05
3rd week	0·04	0·05
4th week	0·00	0·06
5th week	0·00	0·02
6th week	0·00	0·00

A series of experiments on similar lines was carried out on swedes previously killed by ether and washed free from soluble pectin. As with the experiments on apple material a sample left standing with water showed a steady development of soluble pectin. Another portion was boiled with water for three hours and then pressed out and examined. Much soluble pectin was found in the extract. The residue was washed free and then again boiled for another three hours. No soluble pectin was obtained in this case. The residue was divided into two parts for different treatment. One portion was autoclaved with a solution of $N/20$ HCl at 110° and subsequent examination showed that a great deal of pectin had been produced in the process. The second portion was kept in water for one month but no soluble pectin was developed. It was then autoclaved with acid and a great deal of pectin was produced.

The foregoing observations show that a certain amount of pectin appears, if a residue previously washed free from pectin is left with water for a time, but that a steady state is finally reached when no more pectin is produced however long the sample is kept. Similar results are obtained by boiling; a certain amount of pectin is brought into solution but subsequent boiling for long periods has no further effect and no more pectin is developed after prolonged standing with water. On the other hand in both these cases large quantities of pectin are produced by autoclaving with acid. A great deal of further investigation is necessary before any definite conclusions can be made, but such facts as have already been ascertained support the theory that the

production of soluble pectin is due to the action of an enzyme which must be supposed to be present in the cell walls in a form which it was not possible to extract.

SUMMARY.

An account is given of the development of soluble pectin in different kinds of apples kept in cold storage and at ordinary temperature. It is shown that the pectin reaches a maximum during the process of ripening and then gradually falls as the apple becomes over-ripe. The date of picking of the fruit has no effect on the development of the pectin in either cold or ordinary store.

A method is described for the quantitative estimation of protopectin by hydrolysis with weak acid. Preliminary work carried out by the application of this method suggests that there is a definite relationship between the amounts of soluble and insoluble pectin constituents. The experiments indicate the possibility of a third source of pectin.

Preliminary experiments tend to show that the development of the soluble pectin may be attributed to enzyme activity.

This investigation has been carried out for the Food Investigation Board of the Scientific and Industrial Research Department. The author wishes to express her gratitude to Dr Haynes of this Department for her valuable criticism and advice.

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